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(57) Abstract

Melanoma-specific, A1-restricted CTL epitopes have been identified in tyrosinase, and may be used in conjunction with other A1-, A2- and/or A3-restricted epitopes of tyrosinase, pMel-17 and other melanoma antigens in the design of vaccines.

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PEPTIDES RECOGNIZED BY MELANOMA-SPECIFIC A1-, A2- AND A3-RESTRICTED CYTOTOXIC LYMPHOCYTES, AND USES THEREFOR

Priority is claimed from U.S. Ser. No. 60/027,627, filed October 4, 1996, and U.S. Ser. No. 60/013,972, filed March 19, 1996, both incorporated by reference in their entirety.

CROSS-REFERENCE TO RELATED APPLICATIONS

See PCT/US95/01991, filed February 16, 1995, Ser. No. 08/234,784, filed April 29, 1994, now pending, Ser. No. 08/197,399 filed February 16, 1994, now pending, all hereby incorporated by reference in their entirety.

Mention of Government Grant

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BACKGROUND OF THE INVENTION

Field of the Invention

The present invention is directed to peptides that, in association with Class I MHC molecules, form epitopes recognized by cytotoxic T-cells specific for human melanoma, to immunogens comprising said epitopic peptides, and to related compositions, methods and apparatus.

Description of the Background Art

Melanoma affects 30,000 new patients per year in the United States. It is a cancer manifested by the unabated proliferation of melanocytes. Eighty percent of melanoma patients are diagnosed during their productive years between the ages of 25 and 65. The incidence of melanoma is rapidly increasing, in 1935 the lifetime risk of developing melanoma was 1:1,500 individuals, at present, the risk has risen to 1:105. It is believed that by the year 2000 the risk of developing melanoma will increase to about 1:70 to 1:90. Early diagnosis and treatment of this disease is crucial. Once a primary tumor becomes metastatic the disease is almost always fatal.

Cytotoxic lymphocyte (CTL) response has been shown to be an important host defense against malignant cells, Rock et

al. J. Immunol., (1993), 150:1244.

Lymphocytes isolated from patients having melanoma, when stimulated in vitro with recombinant interleukin-2 (rIL-2) and autologous melanoma cells, develop a melanoma specific cytotoxic response, Vose et al., Nature, (1982), 296:359; Knuth et al., Proc. Natl. Acad. USA, (1984), 81:3511; Slingluff et al., Arch. Surg., (1987), 122:1407; Darrow et al., Cancer, (1988), 62:84; Slingluff et al., J. Natl. Cancer Inst., (1988), 80:1016; Slingluff et al., Ann. Surg., (1989), 210:194; Muul et al., J. Immunol., (1987), 138:989; Van den Eynde et al., Int. J. Cancer, (1989), 44:634; Anichini et al., Int. J. Cancer, (1985), 35:683. The majority of melanoma-specific effector lymphocytes are $CD8^+$ cytotoxic T lymphocytes (CTL) that are restricted by class I Major Histocompatibility Complex (MHC) molecules, Vose et al; Slingluff et al (1988), supra, Hersey et al., Cancer Immunol. Immunother., (1986), 22:15. These characteristics are present whether CTL have been generated from peripheral blood lymphocytes (PBL), lymph node cells, or tumor infiltrating lymphocytes.

The evidence that the CTL response to human melanoma is restricted by class I MHC molecules includes demonstration of cross-reactivity for allogenic melanoma cells that share a restricting class I MHC molecule with the autologous tumor. The HLA-A2 molecule and its variants, of which HLA-A2.1 is by far the most common, is an effective restricting element for the melanoma-specific CTL response. Additionally, melanoma-specific HLA-restricted CTL lyse the majority of $A2^+$ melanomas tested, Darrow et al., J. Immunol., (1989), 142:3329; Wolfel et al., J. Exp. Med., (1989), 170:797; Hom et al., J. Immunother., (1991), 3:153. By demonstrating lysis of A2- melanomas transfected with the A2.1 gene, it has been shown that these transfected melanomas can present the epitopes recognized by A2-restricted melanoma-specific CTL, Kawakami et al., J. Immunol., (1992), 148:638. These results suggest that these CTL recognize A2-restricted epitopes that are shared by the majority of melanomas, although very little is known about the number and identity of their epitopes.

Class I molecules of the Major Histocompatibility Complex (MHC) bind to peptides derived from intracellular pathogens or from proteins expressed in tumor cells, and present them on the cell surface to the host immune system. The mechanism of peptide presentation involves protein synthesis and proteolysis in the cytosol, followed by transport of peptides into the endoplasmic reticulum (ER), through the action of the TAP transporter molecules. Peptides then become associated with newly synthesized class 1 molecules, and the resulting complexes move to the cell surface. Proteins that are membrane associated or secreted contain signal sequences that cause them to be contranslationally transferred into the ER from membrane-bound ribosomes. Such proteins would thus be protected from the action of cytoplasmic proteases. However, since peptide epitopes do arise from such proteins, although their TAP dependent expression is unclear, it has been assumed that the proteolysis to generate these peptide epitopes occurs after these proteins have been aberrantly translated on cytoplasmic ribosomes.

Adoptive transfer of tumor stimulated CTL has been associated with some tumor regressions, Rosenberg et al., N. Eng. J. Med., (1988), 319:1676.

An alternate approach to augmenting the T-cell response to melanoma is the use of a vaccine to stimulate CTL in vivo (active specific immunotherapy). Epitopes for $CD8^+$ CTL are believed to be short, usually 9- residue peptides that bind to a cleft on the surface of the class I MHC molecule, Udaka et al., Cell, (1992), 69:989; VanBleek et al., Nature, (1990), 348:213; Falk et al., J. Exp. Med., (1991), 174:425. These peptides, generated from proteolysis of endogenous proteins in the cytosol, are transported to the endoplasmic reticulum, where they become associated with newly synthesized class I MHC molecules. They are then transported to the cell surface, Elliott et al., Nature, (1990), 3348:195. CTL epitopes have been reconstituted in vitro by allowing exogenous peptides to bind to MHC molecules on the cell surface of target cells, Townsend et al., Annu. Rev.

Immunol., (1989), 7:601. However, because of the complexity of the peptide mixture associated with class I MHC molecules, Hunt et al., Science, (1992), 255:1261, the definition of individual peptides that comprise specific CTL epitopes has proven extremely difficult.

One method has been to generate genomic or cDNA libraries from tumor cells followed by transfection of progressively smaller subsets of these molecular clones into cells that express the appropriate MHC molecule, but not the tumor specific epitope. Molecular clones that encode T cell epitopes are identified by their ability to reconstitute tumor-specific T cell recognition of the transfected cells. The exact T cell epitope is then identified by a combination of molecular subcloning and the use of synthetic peptides based on the predicted amino acid sequence. See, e.g., P. van der Brugge, et al., <u>Science</u> 254, 1643 (1991); C. Traversari, et al., <u>J. Exp. Med.</u> 176, 1453 (1992); B. Gaugler, et al., <u>ibid.</u> 179, 921 (1994); T. Boon, et al., <u>Annu. Rev. Immunol.</u> 12, 337 (1994); A.B.H. Baker, et al., <u>J. Exp. Med.</u> 179, 1005 (1994); Y. Kawakami, et al., <u>Proc. Natl. Acad. Sci. USA</u> 91, 6458 (1994); P.G. Coulie, et al., <u>J. Exp. Med.</u> 180, 35 (1994); Y. Kawakami, et al., <u>ibid.</u> 180, 347 (1994); V. Brichard, et al., <u>ibid.</u> 178, 489 (1993); T. Wolfei, et al., <u>Eur. J. Immunol.</u> 150, 2955 (1993). Unfortunately, it is possible to inadvertently identify clones that encode cross-reacting peptides that are recognized because of their high level of expression in the transfectants.

By this genetic method, an HLA-A1 restricted T cell epitope (EADPTGHSY) of a melanoma-associated antigen, MAGE-1, was identified. Traversari, et al., <u>J. Exp. Med.</u>, 176:1453-57 (1992). MAGE-1 is expressed in about 20-40% of cancers of several different tissue types, including melanomas, breast cancers, non-small cell lung cancers, head and neck squamous cell cancers, and bladder cancer. It is also found in the normal male testis. The MAGE gene family also includes another member, MAGE-3, for which a homologous HLA-A1-restricted CTL epitope (EVDPIGHLY) was determined, although

only after the first priority date. HLA-A1-restricted CTL epitopes are of limited utility because only a minority of melanomas are HLA-A1$^+$. The function of the MAGE gene products is not known.

The genetic approach has also been used to identify HLA-A2.1-restricted CTL epitopes on tyrosinase. This enzyme is not tumor-specific; it is expressed by normal melanocytes as well as melanoma cells. Tyrosinase is involved in melanin biosynthesis. Autologous CTL recognized tyrosinase-derived HLA-A2-restricted epitopes (YMNGTMSQV and MLLAVLYCL). See Wolfel, et al., Eur. J. Immunol., 24:759-64 (1994). However, these peptides were not recognized by the other CTL lines tested.

Another tissue-specific protein, gp100, is the target of the antibody HMB45, which is specific for melanoma and melanocytes. Based on the correlation between HMB45 activity and recognition by a single TIL-derived HLA-A2-restricted melanoma-specific CTL line, Bakker, et al., J. Exp. Med., 179:1005-9 (1994) established that transfection of cells with the gene for gp100 reconstituted the epitope recognized by this T cell. A subsequent study, using the same T-cell line to screen transfected cDNA libraries also identified the peptide LLDGTATLRL as being sufficient to reconstitute activity. This study was not published prior to Applicants' first priority date. Gp100 is believed to play a role in melanin biosynthesis.

An HLA-A2.1-restricted epitope (AAGIGILTV) has also been identified genetically in another melanocytic protein, MART-1 (Melan-A). Kawakami, et al., J. Exp. Med., 180:347-52 (1994) and Proc. Nat. Acad. Sci. USA, 91:3515-19 (1994), and see also Coulie, et al., J. Exp. Med., 180:35-42 (1994).

An alternate approach toward characterization of CTL epitopes is to identify them directly. Naturally occurring peptides associated with MHC molecules on the tumor cells are directly extracted, fractionated by HPLC and used to reconstitute recognition by tumor specific CTL of a non-tumor cell expressing appropriate MHC molecules. Sequencing can be performed by Edman degradation. Mandelboim, et al., Nature,

369:67-71 (1994) (CTL epitope on murine lung carcinoma). However, Applicants pioneered the use of tandem mass spectrometry to evaluate HHC-associated peptides. C.L. Slingluff, et al., J. Immunol. 150, 2955 (1993); D.F. Hunt, et al., Science 255, 1261 (1992); R.A. Henderson, et al., Proc. Natl. Acad. Sci. USA 90, 10275 (1993).

However, when peptides associated with MHC molecules on tumor cells are extracted, a complex mixture, of up to 10,000-20,000 different peptides of similar size (mostly nonamers), is obtained. Within this mixture, only a small number of molecules are likely to correspond to the peptides of interest. Consequently, their isolation and sequencing was extremely difficult. Boon, et al., Ann. Rev. Immunol., 12:337-65 (1994) states, "to our knowledge, the peptide elution method has not yet ensured the identification of a peptide recognized by anti-tumor CTL". More colorfully, Finn, et al., Curr. Op. Immunol., 5:701-8 (1993) likened the process to "throwing a fish hook into the ocean, hoping to catch the big one", given, inter alia, the "very low amounts of peptides".

In the present invention, HLA associated peptides have been extracted, isolated and identified from different melanoma lines. These peptides can be used to reconstitute epitopes for HLA-A2.1- and HLA-A3- restricted melanoma-specific CTL. These peptides and the stimulated CTL may be useful for the in vivo immunotherapeutic treatment of melanoma. Aspects of applicants' invention were described in Cox, et al., Science, 264:716-719 (1994), which was published on April 29, 1994.

SUMMARY OF THE INVENTION

The present invention relates to immunogens which are capable of eliciting a melanoma-specific cytotoxic lymphocyte response in at least some individuals, which response is directed to peptide epitopes carried by those immunogens, and to the use of those immunogens in active specific immunotherapy and immunoprophylaxis against melanoma.

These immunogens may be used as vaccines, in active specific immunotherapy. The immunogens may be administered directly or by gene therapy. The epitopic peptides may also be used to stimulate lymphocytes, the latter then being used for adoptive immunotherapy.

In one embodiment, a CTL epitope of the present invention is a sequence which is at least substantially homologous with a CTL epitope of the melanoma antigens pMel-17 and gp100, (these two antigens are essentially identical). One such epitope is the peptide 946L. Peptide 946I is substantially homologous to peptide 946L.

In another embodiment, a CTL epitope of the present invention is a sequence which is at least substantially homologous with a CTL epitope of tyrosinase. One such epitope is the peptide Lys-Cys-Asp-Ile-Cys-Thr-Asp-Glu-Tyr. Peptides 946I and 946L, related to a single segment in pMel-17 (a protein homologous to gp100), had unexpectedly high A2.1 CTL stimulatory activity. They also are recognized by CTL from different individuals.

Another pMel-17-derived peptide (ALLAVGATK) had acceptable A3 CTL stimulatory activity, and is the first HLA-A3-associated stimulatory peptide identified in pMel-17 and one of the few, if any, A3-associated peptides identified in melanoma antigens generally.

KCDICTDEY is the first A1-restricted epitope to be identified in tyrosinase and one of the few such epitopes identified in melanoma antigens generally (A1 epitopes have been identified in MAGE-1 (EADPTGHSY) and MAGE-3 (EVDPIGHLY)).

It is advantageous to be able to elicit a melanoma-specific CTL response from one or more A1-, A2.1- and/or A3-

Figure 5.restricted CTLs, and preferably all of them. In a similar manner, a melanoma-specific CTL response may be elicited which is restricted by other MHC molecules.

Additional embodiments of the present invention are described below.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A Melanoma specific recognition of autologous tumor by VMM18 CTL. VMM18 cells (solid squares) were lysed by the CTL in a 4 h ^{51}Cr release assay, while minimal lysis of non-melanoma targets K562 (open squares), VMM12-EBV (open circles) and the HLA-A3$^-$ melanoma DM6 (open triangles) was observed.

Figure 1B Recognition of VMM18 melanoma by VMM18 CTL was restricted by the class I MHC molecule HLA-A3. Lysis of autologous melanoma was inhibited after incubation of target cells with W6/32 (solid diamonds) and GAP-A3 (solid squares) MAbs, specific for class I MHC and HLA-A3 respectively. Incubation with L243 (open circles) had little effect on recognition of autologous melanoma. Specific lysis of autologous melanoma was 65% (dotted line), while lysis of VMM12-EBV was 1.5% (solid line). The effector:target ratio used was 10:1.

Figure 2 VMM18 CTL recognize a shared antigen expressed by HLA-A3$^+$ melanomas. Lysis of hot (^{51}chromium labeled) autologous and HLA-A3$^+$ allogeneic melanoma cells (see legend) was inhibited by cold (unlabelled) VMM18 melanoma cells (top fig.), but not by cold (unlabelled) HLA-A3$^-$ DM6 melanoma cells (bottom fig.). 2×10^4 VMM18 CTL were incubated with 1.4×10^4 unlabelled (cold) VMM18 or DM6 melanoma cells for 1 h at 37°C, prior to the addition of 2×10^3 ^{51}Cr-labelled targets as indicated, giving a final E:T ratio of 10:1.

Figure 3 Expression of Pmel-17 reconstitutes recognition of non-melanoma HLA-A3$^+$ target cells by VMM18 CTL. VMM18 CTL lysed ^{51}Cr-labeled autologous melanoma cells VMM18 (solid squares) as well as a non-melanoma HLA-A3$^+$ cell line VMM12-EBV infected with recombinant vaccinia virus expressing Pmel-17 (vac-Pmel-17, closed circles). Minimal lysis of uninfected VMM12-EBV cells (open circles), or cells infected with control recombinant vaccinia virus expressing influenza nucleoprotein (vac-NP, open triangles), was observed.

Figure 4 Relative ability of Pmel-17 peptides to sensitize non-melanoma target cells for recognition by VMM18 CTL. ^{51}Cr-labelled T2-A3 cells were incubated with Pmel-17

peptides ALLAVGATK (solid squares) and LLAVGATK (solid triangles) and the control HLA-A3 binding peptide QVPLRPMTYK, from the HIV Nef protein (open circles).

Figures 5A-B. Recognition of autologous and HLA-matched melanomas by melanoma-reactive CTL. In 19**A**), VMM12 CTL are evaluated for lysis of a panel of target cells. The VMM12 CTL recognize shared melanoma antigens presented by HLA-A1 (VMM15 melanoma cells share HLA-A1 with VMM12), and by HLA-A3 (VMM10 melanoma cells share HLA-A3 with VMM12). Similarly, in 19**B**), VMM15 CTL are evaluated in the same manner. VMM15 CTL recognize shared melanoma antigens presented by HLA-A1 (VMM12 melanoma cells) and by either HLA-A1, -A25, or -B8 (VMM14 melanoma cells).

Figures 6A-B. HLA-A1+ CTL lines recognize tyrosinase peptides on HLA-A1. In 20**A**), VMM12 CTL are capable of lysing C1R-A1 cells infected with a vaccinia-tyrosinase construct. In 20**B**), VMM15 CTL also recognize tyrosinase.

Figures 7A-D. List of peptides synthesized and tested for recognition by VMM12 and VMM15 CTL. These peptides were predicted from the defined sequence of tyrosinase, accounting for some possible alternate sequences and for possible post-translational modifications. Those listed in the 3rd synthesis were not tested. Figs. 21A-D refers to syntheses 1-4, respectively.

Figure 8. VMM15 CTL recognize peptides containing KCDICTDEY in association with HLA-A1. C1R-A1 cells were pulsed with 10 uM, 1 uM and 0.1 uM concentrations of synthetic peptides prior to addition of VMM15 CTL. Background lysis of C1R was approximately 10%. Direct cytotoxicity by the peptides themselves was negligible (open diamonds), averaging 0-2%. An epitope for VMM15 CTL was reconstituted by three of the test peptides, numbers 5, 12, and 15, corresponding to KCDICTDEY, DAEKCDICTDEY, and EKCDICTDEY as marked.

Figure 9. VMM12 CTL recognize a peptide containing KCDICTDEY in association with HLA-A1. C1R-A1 cells were pulsed with peptides at 1 to 0.01 uM concentrations prior to adding VMM12 CTL. The peptides themselves were not cytolytic

(open diamonds). The peptide DAEKCDICTDEY reconstituted an epitope for these VMM12 CTL, although weakly.

Figure 10. Amino acid sequence of tyrosinase, with the position of KCDICTDEY highlighted and underlined. The high proportion of cystine residues and acidic residues are noted relative to the proportion in the intact protein.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS OF THE INVENTION

The present invention relates to certain melanoma-specific CTL epitopes, and their incorporation into immunogens for immunoprophylactic and immunotherapeutic purposes. For the purpose of the present invention, a melanoma-specific CTL epitope is an epitope which is recognized by a T-cell receptor of at least some cytotoxic lymphocytes of at least some individuals in the population of interest, and which is more frequently or strongly associated with melanoma cells than with at least some other cancer and/or normal cells. There may be some cross-reactivity, for example, with other cells of melanocytic lineage. Absolute specificity is not required, provided that a useful prophylactic, therapeutic or diagnostic effect is still obtained.

Melanoma-Specific CTL Epitopes

The melanoma-specific CTL epitopes of the present invention are peptides, typically 9-13 amino acids in length, which are sufficiently similar to a melanoma-specific epitope recognized by a melanoma-specific CTL to be useful, under suitable conditions of use, to protect an individual from melanoma, or to be useful in the diagnosis of melanoma or of a patient's ability to fight a melanoma by a CTL response. Preferably, these epitopes are identical to or otherwise substantially homologous with melanoma-specific peptide epitopes recognized by melanoma-specific CTLs.

The family of melanoma epitopes which are recoverable from an individual is dependent on the nature of the binding site of the Class I MHC (HLA) molecules expressed by the individual, and, as a result of the polymorphism of the Class I MHC (HLA) molecules, can vary considerably from one individual to another. For the purpose of the present invention, the melanoma cell line used as a source of melanoma-specific CTL epitopes may be any melanoma cell line; similarly, the Class I MHC (HLA) molecule may be any such molecule borne by a melanoma which is capable of binding to and presenting a melanoma-specific epitope, including, but

not limited to, the various allelic forms of Class I MHC molecules, including but not limited to those enumerated in Table I. Among the Class I molecules, the principal genetic loci are denoted as HLA-A, HLA-B, and HLA-C. The preferred epitopic sequence may vary depending on the restriction system.

Application of active specific immunotherapy to a heterogeneous melanoma patient population would be facilitated by identification of CTL epitopes presented in association with a wide range of class I MHC molecules. Besides HLA-A2, the most commonly expressed class I MHC molecules are A1 and A3, then B7 and B8. Approximately 90% of the melanoma patient population should express one or more of these molecules or HLA-A2. Peptides from MAGE-1 and MAGE-3 have been identified as HLA-A1-restricted CTL epitopes, and a few peptides have been identified for some of the less common MHC molecules, including A24, A31, and B44. Little work has been done toward identification of HLA-A3-restricted responses, and-except for the peptides from MAGE proteins - little work has been done toward identification of HLA-A1-restricted responses.

Preferably, the epitope is one restricted by one of the more prevalent forms (in the melanoma patient population) of these loci. The loci HLA-A1, HLA-A2, HLA-A3, HLA-B7 and HLA-B8 are of greatest interest. Within HLA-A2, HLA-A2.1 is of particular interest.

Preferably, the CTL epitopes of the present invention, in the cytotoxicity assay described hereafter, when used in oligopeptide form to reconstitute epitopes for suitable CTL, achieve, at the dosage resulting in maximal lysis of target cells exposed to the stimulated CTL, a percentage lysis of target cells which is at least 10 percentage points higher (more preferably, at least 20 points higher) the background level of lysis of the target cells by the CTLs (i.e., in absence of the peptide).

Preferably, the peptide concentration at which the epitope-stimulated CTLs achieve half the maximal increase in lysis relative to background is no more than about 1 mM,

preferably no more than 1μM, more preferably no more than about 1 nM, still more preferably no more than about 100 pM, most preferably no more than about 10 pM. For the peptides 946L and 946I, half-maximal lysis of T2 cells is observed with concentrations of peptide in the pM range. In contrast, the MAGE-1 peptide EADPTGHSY had half-maximal lysis between 1 and 100 nM (prob about 10); while the tyrosinase peptides YMNGTMSQV and MLLAVLYCL reported by Boon induced half-maximal lysis (even with pre-treatment with MA2.1 antibody) at over 10 nM.

ALLAVGATK is at present the only pMel-17 derived peptide known to be immunogenic in the context of HLA-A3, which is expressed by 20% of the patient population. It achieves half-maximal lysis of T2 cells expressing HLA-A3 at a concentration of about 10 nM. While not as potent as our A2.1 peptides, its potency is acceptable.

Preferably the epitope is recognized by CTLs from at least two different individuals, more preferably at least five different individuals.

More preferably, the CTL epitope satisfies two or more of the above desiderata.

The 946L peptide, although recognized by HLA-A2.1-restricted melanoma-specific CTL, may not be optimal at present. It is known that some residues on the nonamer peptide are particularly important for binding of the peptide to the MHC molecule (residues 2,9), while others are particularly important for Tc recognition (residues 4-8). The other residues may be important for either or both. It is proposed that amino acid substitutions for the 946 peptide may be useful at increasing immunogenicity, particularly by attempting to change residues that may increase binding to the MHC such as changing residue 9 to a valine or residue 3 to anything other than glutamic acid (E). Using existing knowledge about which of these residues may be more likely to affect binding either to the MHC or to the TcR, a rational approach to this process may be employed. The resulting peptides, if more effective, could be used for any of the purposes described herein. (refs: E.L. Huczko et al. J.

Immunol. 151:2572, 1993; J. Ruppert et al. Cell 754: 929, 1993; Madden Dr et al. Cell 75:693-708, 1994.) It is possible to predict peptides binding to specific Class I MHC molecules by identifying amino acid sequences fitting described binding motifs within known protein sequences. In attempting to identify epitopes for melanoma-specific CTL, these peptides can be screened for their ability to sensitize non-melanoma targets for recognition by melanoma specific CTL.

Therefore, in addition to epitopes which are identical to the naturally occurring melanoma-specific epitopes, the present invention embraces epitopes which are substantially homologous with such epitopes, and therefore melanoma-specific in their own right.

The term "substantially homologous", when used in connection with amino acid sequences, refers to sequences which are substantially identical to or similar in sequence with each other, giving rise to a homology in conformation and thus to similar (or improved) biological activity. The term is not intended to imply a common evolution of the sequences.

An epitope is considered substantially homologous to a reference epitope if it has at least 10% of an immunological activity of the reference epitope and differs from the reference epitope by no more than one non-conservative substitution not suggested by a known binding motif of the pertinent MHC molecule. Any number of highly conservative, conservative or semi-conservative substitutions, or non-conservative substitutions suggested by known binding motifs, subject to the activity limitation, are permitted.

Kast, et al., J. Immunol, 152:3904-12 (1994) sets forth HLA-A specific peptide binding motifs for the HLA molecules A1, A2.1, A3, A11 and A24. Engelhard, et al., in Sette, ed., Naturally Processed Peptides, 57:39-62 (1993) explored the features that determined binding to HLA-A2.1 and HLA-B7. See also Hobohim et al; Eur. J. Immunol., 23:1271-6 (1993); Kawakami, et al., J. Immunol., 154:3961-8 (1995). Based on these and other sources, the preferred and tolerated AAs for

various HLA molecules include (but are not limited to) the following:

Table 10

Molecule	Position	Preferred AA	tolerated AA
A1	2	T, S, M	
	3	D, E	A, S
	9	Y	
A2.1	2	L, M	I, V, A, T
	9	L, V, I	A, M, T
A3	2	L, M, I, V, S A, T, F	C, G, D
	9	K, R, Y, H, F	A
A11	2	M, L, I, V, S A, T, G, N	C, D, F
	9	K	R, H, Y
A24	2	Y, F, W	M
	9	F, L, I, W	
B7	1	A	M, S, R, L V
	2	P	
	3	R	A, K, S, M
	9	L	I, A, V
B8	3	K	not known
	5	K	not known
	9	L	not known
B27	2	R	not known
	9	R, K, H	not known
B35	2	P	not known
	9	Y	not known
B53	2	P	not known

If a position is not listed, studies revealed a greater variability of AAs than for the listed positions. For listed positions, AAs not listed may be tolerated, especially if they are conservative or semi-conservative substitutions for "preferred" or "tolerated" AAs.

An example of a peptide variant which satisfies the known binding motif is YLEPGPVTV. This differs from 946L at position 9. However, V is a preferred a.a. at position 9 of

HLA-A2.1 binding peptides.

Substantially homologous peptide epitopes may be identified by a variety of techniques. It is known in the art that one may synthesize all possible single substitution mutants of a known peptide epitope. For a nonpeptide, there are (20x9-1=179) such mutants. Geysen, et al., Proc Nat. Acad. Sci. (USA), 81:3998-4002 (1984). While the effects of different substitutions are not always additive, it is reasonable to expect that two favorable or neutral single substitutions at different residue positions in the epitope can safely be combined in most cases.

One may also synthesize a family of related single or multiple substitution mutants, present the mixture to the HLA-A2.1 positive lymphoblastoid cell line T2 (or other cell line capable of presenting melanoma-specific CTL epitopes), and expose the T2 cells to melanoma-specific CTLs. If the T2 cells are lysed, the effective epitopes may be identified either by direct recovery from the T2 cells or by a progressive process of testing subsets of the effective peptide mixtures. Methods for the preparation of degenerate peptides are described in Rutter, USP 5,010,175, Haughten, et al., Proc. Nat. Acad. Sci. (USA), 82:5131-35 (1985), Geysen, et al., Proc. Nat. Acad. Sci. (USA), 81:3998-4002 (1984); W086/06487; W086/00991.

Multiple mutagenesis may be used to screen a few residue positions intensely or a larger number of positions more diffusely. One approach is to explore at least a representative member of each a.a. type at each position, e.g., one representative of each of exchange groups I-V as hereafter defined. Preferably, Gly and Pro are screened in addition to one other group I residue. Preferably, at least one screened residue is an H-bonding resiude. If a positive mutant features a particular representative, like amino acids can be explored in a subsequent library. If, for example, a Phe substitution improves binding, Tyr and Trp can be examined in the next round.

In the case of the peptide 946L (SEQ. ID. No.:14), a possible multiple mutagenesis strategy would be as follows:

Parental	**Tyr**	**Leu**	**Glu**	**Pro**	**Gly**	**Pro**	**Val**	**Thr**	**Ala**
Possible Mutations	Phe	Ile	Asp	Ala	Pro	Ala	Ile	Ala	Thr
	Trp	Val		Ser	Ala	Ser	Leu	Ser	Ser
		Met		Thr	Ser	Thr	Met	Pro	Pro
		Ala		Gly	Thr	Gly		Gly	Gly
		Thr							Leu
									Val
									Ile
									Met

For peptide 1030, a possible strategy would be:

Parental	Tyr	Met	Asp	Gly	Thr	Met	Ser	Gln	Val
	Phe	Val	Glu	Pro	Ala	Val	Ala	Asn	Ile
	Trp	Ile		Ala	Ser	Ile	Thr		Leu
		Leu		Ser	Pro	Leu	Pro		Met
		Ala		Thr	Gly		Gly		Ala
		Thr							Thr

Other strategies are, of course, possible. For example, the Asp/Glu and Gln/Asn sets can be merged. It is known from comparison of peptide 1030 with the homologous tyrosinase segment that substitution of Asn for Asp in position 3 reduces CTL activity 100-fold. However, a multiple mutagenesis strategy could identify compensating mutations at other sites.

For our preferred A3 peptide, a possible multiple mutagenesis strategy would be

Ala	Leu	Leu	Ala	Val	Gly	Ala	Thr	Lys
Thr	Ile	Ile	Thr	Ile	Ala	Thr	Gly	Arg
Ser	Val	Val	Ser	Leu	Thr	Ser	Ala	His
Pro	Met	Met	Pro	Met	Ser	Pro	Ser	Tyr
Gly	Ser		Gly		Pro	Gly	Pro	Phe
	Cys							Ala
	Gly							
	Asp							
	Ala							
	Thr							
	Phe							

For our preferred A1 peptide, a possible multiple mutagenesis strategy would be

Lys	Cys	Asp	Ile	Cys	Thr	Asp	Glu	Tyr
Arg	Thr	Glu	Leu	Thr	Ala	Glu	Asp	Phe
His	Ser		Val	Ser	Ser			Trp
	Ala		Met	Ala	Pro			
	Met			Gly	Gly			

These strategies take into account both conservative substitutions for the wild type AAs, and the known A1, A2.1 and A3 binding motifs.

The person of ordinary skill in the art, in determining which residues to vary, may also make comparisons of the sequences of the naturally processed MHC associated peptides, and may obtain 3D structures of the MHC: peptide: TCR complexes, in order to identify residues involved in MHC or TCR binding. Such residues may either be left alone, or judiciously mutated in an attempt to enhance MHC or TCR binding.

It is also possible to predict substantially homologous epitopes by taking into account studies of sequence variations in families of naturally occurring homologous proteins. Certain amino acid substitutions are more often tolerated than others, and these are often correlatable with similarities in size, charge, etc. between the original amino acid and its replacement. Insertions or deletions of amino acids may also be made. N- and C-terminal truncations or extensions are more likely to be tolerated than internal deletions or insertions. With regard to truncation, the peptide may be truncated by one or more amino acids and still be substantially homologous, however, it cannot be fewer than five amino acids. Extensions are permissible, however, note that larger peptides are digested in vivo prior to presentation.

Conservative substitutions may be made in the amino acid sequence of the proteins of interest without compromising the desired properties of the peptides, i.e., induction of cytotoxic T-lymphocytes in a patient when administered thereto.

Conservative substitutions are herein defined as exchanges within one of the following five groups:

I. Small aliphatic, nonpolar or slightly polar residues:
Ala, Ser, Thr, Pro, Gly

II. Polar, negatively charged residues: and their amides
Asp, Asn, Glu, Gln

III. Polar, positively charged residues:
His, Arg, Lys

IV. Large, aliphatic, nonpolar residues:
Met, Leu, Ile, Val, Cys

V. Large, aromatic residues:
Phe, Tyr, Trp

Within the foregoing groups, the following substitutions are considered "highly conservative":

Asp/Glu
His/Arg/Lys
Phe/Tyr/Trp
Met/Leu/Ile/Val

Semi-conservative substitutions are defined to be exchanges between two of groups (I)-(V) above which are limited to supergroup (A), comprising (I), (II) and (III) above, or to supergroup (B), comprising (IV) and (V) above. Also, Ala is considered a semi-conservative substitution for all non group I amino acids.

It will be appreciated that highly conservative substitutions are less likely to affect activity than other conservative substitutions, conservative substitutions are less likely to affect activity than merely semi-conservative substitutions, and semi-conservative substitutions less so than non-conservative substitutions.

Although a substitution mutant, either single or multiple, of the peptides of interest may not have quite the potency of the original peptide, such a mutant may well be useful.

Substitutions are not limited to the genetically encoded, or even the naturally occurring amino acids. When the epitope is prepared by peptide synthesis, the desired amino acid may be used directly. Alternatively, a genetically encoded amino acid may be modified by reacting it with an organic derivatizing agent that is capable of reacting with selected side chains or terminal residues. The following

examples of chemical derivatives are provided by way of illustration and not by way of limitation.

Aromatic amino acids may be replaced with D- or L-naphylalanine, D- or L-Phenylglycine, D- or L-2-thieneylalanine, D- or L-1-, 2-, 3- or 4-pyreneylalanine, D- or L-3-thieneylalanine, D- or L-(2-pyridinyl)-alanine, D- or L-(3-pyridinyl)-alanine, D- or L-(2-pyrazinyl)-alanine, D- or L-(4-isopropyl)-phenylglycine, D-(trifluoromethyl)- phenylglycine, D-(trifluoromethyl)-phenylalanine, D-p-fluorophenylalanine, D- or L-p-biphenylphenylalanine, D- or L-p-methoxybiphenylphenylalanine, D- or L-2-indole-(alkyl)alanines, and D- or L-alkylainines where alkyl may be substituted or unsubstituted methyl, ethyl, propyl, hexyl, butyl, pentyl, iso-propyl, iso-butyl, sec-isotyl, iso-pentyl, non-acidic amino acids, of C1-C20.

Acidic amino acids can be substituted with non-carboxylate amino acids while maintaining a negative charge, and derivatives or analogs thereof, such as the non-limiting examples of (phosphono)-alanine, glycine, leucine, isoleucine, threonine, or serine; or sulfated (e.g., $-SO_3H$) threonine, serine, tyrosine.

Other substitutions may include unnatural hyroxylated amino acids made by combining "alkyl" (as defined and exemplified herein) with any natural amino acid. Basic amino acids may be substituted with alkyl groups at any position of the naturally occurring amino acids lysine, arginine, ornithine, citrulline, or (guanidino)-acetic acid, or other (guanidino)alkyl-acetic acids, where "alkyl" is define as above. Nitrile derivatives (e.g., containing the CN-moiety in place of COOH) may also be substituted for asparagine or glutamine, and methionine sulfoxide may be substituted for methionine. Methods of preparation of such peptide derivatives are well known to one skilled in the art.

In addition, any amide linkage can be replaced by a ketomethylene moiety, e.g. $(-C(=O)-CH_2-)$ for $(-(C=O)-NH-)$. Such derivatives are expected to have the property of increased stability to degradation by enzymes, and therefore possess advantages for the formulation of compounds which may

have increased in vivo half lives, as administered by oral, intravenous, intramuscular, intraperitoneal, topical, rectal, intraocular, or other routes.

In addition, any amino acid can be replaced by the same amino acid but of the opposite chirality. Thus, any amino acid naturally occurring in the L-configuration (which may also be referred to as the R or S configuration, depending upon the structure of the chemical entity) may be replaced with an amino acid of the same chemical structural type, but of the opposite chirality, generally referred to as the D-amino acid but which can additionally be referred to as the R- or the S-, depending upon its composition and chemical configuration. Such derivatives have the property of greatly increased stability to degradation by enzymes, and therefore are advantageous in the formulation of compounds which may have longer in vivo half lives, when administered by oral, intravenous, intramuscular, intraperitoneal, topical, rectal, intraocular, or other routes.

The thiol group of cysteine reacts very rapidly with alkyl halides, such as iodoacetate, iodoacetamide, methyl iodine, and so on, to give the corresponding stable alkyl (substituted or unsubstituted) derivatives, such as $-CH_2-S-CH_3$. The thiol group can also add across double bonds such as those of N-ethylmaleimide or of maleic anhydride, and it can open the ring of ethyleneimine, providing a new site for tryptic cleavage. Thiols form complexes with various metal (especially mercury, silver, arsenic, copper, iron, zinc, cobalt, molybdenum, manganese and cadmium ions) and organometal ions (e.g., $R-Hg^+$, such as para-mercuribenzoic acid).

The thiol group may be oxidized to yield a disulfide bond or a sulfonate. A thiol may be converted to a disulfide by thiol-disulfide exchange, for example, exchange with an aromatic disulfide such as dithionitrobenzoic acid (DTNB) or Ellman's reagnet. Of course, a cysteine residue may be disulfide bonded to a cysteine residue in the same or a different peptide, or to a free cysteine. By way of further examples, some of which are already embraced by the general

discussion above, cysteinyl residues may be reacted with alpha-haloacetates (and corresponding amines), such as 2-chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteinyl residues may also be derivatized by reaction with compounds such as bromotrifluoroacetone, alpha-bromo-beta-(5-imidozoyl)propionic acid, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

Histidyl residues may be derivatized by reaction with compounds such as diethylprocarbonate e.g., at pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain, and para-bromophenacyl bromide may also be used; e.g., where the reaction is preferably performed in 0.1 M sodium cacodylate at pH 6.0.

Lysinyl and amino terminal residues may be reacted with compounds such as succinic or other carboxylic acid anhydrides. Derivatization with these agents is expected to have the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing alpha-amino-containing residues include compounds such as imidoesters/e.g., as methyl picolinimidate; pyridoxal phosphate; pyridoxal; chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea; 2,4 pentanedione; and transaminase-catalyzed reaction with glyoxylate.

Arginyl residues may be modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin according to known method steps. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high pKa of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine epsilon-amino group.

The specific modification of tyrosyl residues per se is

well-known, such as for introducing spectral labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetranitromethane. N-acetylimidizol and tetranitromethane may be used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively.

Carboxyl side groups (aspartyl or glutamyl) may be selectively modified by reaction with carbodiimides (R'-N-C-N-R') such as 1-cyclohexyl-3-(2-morpholinyl-(4-ethyl) carbodiimide or 1- ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues may be converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

Glutaminyl and asparaginyl residues may be readily deamidated to the corresponding glutamyl and aspartyl residues. Alternatively, these residues may be deamidated under mildly acidic conditions. Either form of these residues falls within the scope of the present invention.

Derivatization with bifunctional agents is useful for cross-linking the peptide to a water-insoluble support matrix or to other macromolecular carriers, according to known method steps. Commonly used cross-linking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'- dithiobis(succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[(p-azidophenyl)dithio]propioimidate yield photoactivatable intermediates that are capable of forming crosslinks in the presence of light. Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. Patent Nos. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 (which are herein incorporated entirely by reference), may be employed for protein immobilization.

Other modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or

threonyl residues, methylation of the alpha-amino groups of lysine, arginine, and histidine side chains (Creighton, T.E., Proteins: Structure and Molecule Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)), acetylation of the N-terminal amine, methylation of main chain amide residues (or substitution with N-methyl amino acids) and, in some instances, amidation of the C-terminal carboxyl groups, according to known method steps. Glycosylation is also possible.

Derivatized moieties may impart altered affinity for their target, altered immunogenicity, or improved solubility, absorption, biological half life, and the like, or attenuated undesirable side effects. Moieties capable of mediating such effects are disclosed, for example, in Remington's Pharmaceutical Sciences, 16th ed., Mack Publishing Co., Easton, PA (1980).

Modifications are not limited to the side chains of the amino acids. One may also modify the peptidyl linkage itself, e.g., -NRCO- (where R is alkyl or aryl), instead of -NHCO-, as in the so-called "peptoids."

The peptides may also comprise isoteres of two or more residues in the immunogenic peptide. An isotere as defined here is a sequence of two or more residues that can be sustituted for a second sequence because the steric conformation of the first sequence fits a binding site specific for the second sequence. The term specifically includes peptide backbone modifications well known to those skilled in the art. Such modifications include modifications of the amide nitrogen, the α-carbon, amide carbonyl, complete replacement of the amide bond, extensions, deletions or backbone crosslinks. See, generally, Spatola, Chemistry and Biochemistry of Amino Acids, peptides and Proteins, Vol. VII (Weinstein ed., 1983).

It is also possible to construct and use so-called peptide mimetics whose conformation is similar to that of a peptide but do not have a peptide-like molecular formula. In effect, in a mimetic, all of the residues of the peptide are replaced by one or more isoteres as defined above.

The Melanoma-Specific Immunogen

The melanoma-specific immunogen of the present invention is a molecule corresponding to or otherwise comprising a melanoma-specific CTL epitope as previously described. The immunogen may comprise one or more melanoma-specific CTL epitopes, which may be the same or different. Preferably, the immunogen is chosen so that at least one epitope is effective in each of two or more restriction systems, e.g., HLA-A1 and HLA-A3; HLA-A1 and HLA-A2; HLA-A2 and HLA-A3; and HLA-A1, -A2 and -A3. In some instances, a single epitope may be effective in more than one restriction system. For example HLA-A2 and HLA-69, or HLA-A3 and HLA-A11, are pairs of MHC molecules having similar peptide binding motifs. Otherwise, for the immunogen to be effective in more than one restriction system, two or more epitopes (at least one for each MHC molecule of interest) will need to be provided. These epitopes may be separate or overlapping.

It should be noted that instead of linking epitopes within a single immunogen, the compositions of the present invention may include two or more immunogens which present different epitopes.

If the immunogen comprises a plurality of such epitopes, they may be linked directly, or through a spacer of some kind, or by noncovalent means such as an avidin:biotin complex. The immunogen may take any form that is capable of eliciting a melanoma-specific cytotoxic immune response. By way of example and not of limitation, the immunogen may be a fusion of a plurality of CTL epitopes which is sufficiently large to be immunogenic, a conjugate of one or more epitopes to a soluble immunogenic macromolecular carrier, such as serum albumin, keyhole limpet hemocyanin, or dextran, a recombinant virus engineered to display the epitope on its surface, or a conjugate of a plurality of epitopes to a branched lysine core structure, a so-called "multiple antigenic peptide" (see Posnett, et al., J. Biol. Chem., 263:1719-25, 1988).

The immunogenic conjugate may also comprise moieties intended to enhance the immune response, such as a T helper

peptide, a cytokine or an adjuvant; a targeting agent, such as an antibody or receptor ligand or ligand analogue; or a stabilizing agent, such as a lipid.

For instance, the ability of the peptides to induce CTL activity can be enhanced by linkage to a sequence which contains at least one epitope that is capable of inducing a T helper cell response. Particularly preferred immunogenic peptides/T helper conjugates are linked by a spacer molecule. The spacer is typically comprised of relatively small, neutral molecules, such as amino acids or amino acid mimetics, which are substantially uncharged under physiological conditions. The spacers are typically selected from, e.g., Ala, Gly, or other neutral spacers of nonpolar amino acids or neutral polar amino acids. It will be understood that the optionally present spacer need not be comprised of the same residues and thus may be a hetero- or homo-oligomer. When present, the spacer will usually be at least one or two residues, more usually three to six residues. Alternatively, the CTL peptide may be linked to the T helper peptide without a spacer.

The immunogenic peptide may be linked to the T helper peptide either directly or via a spacer either at the amino or carboxy terminus of the CTL peptide. The amino terminus of either the immunogenic peptide or the T helper peptide may be acylated.

Besides one or more of the novel melanoma-specific CTL epitopes described herein, the immunogen may present one or more such epitopes already known in the art, such as the following:

Table A. Peptide epitopes for human tumor-specific CTL

Protein	**MHC restriction**	**Peptide sequence**	**Tumor type**
Tyrosinase	A2	MLLAYLYCL	Melanoma
Tyrosinase	A24	AFLPWHRLF, AFLPWHRLFL	Melanoma
Tyrosinase	B44	SEIWRDIDF	Melanoma

gp100/Pmel17	A2	KTWGQYWQV	Melanoma
gp100/Pmel17	A2	ITDQVPFSV	Melanoma
gp100/Pmel17	A2	VLYRYGSFSV	Melanoma
gp100/Pmel17	A2	LLDGTATLRL	Melanoma
MART-1/MelanA	A2	AAGIGILTV	Melanoma
MART-1/MelanA	A2	ILTVILGVL	Melanoma
gp75/TRP-1	A31	----	Melanoma
MAGE-1	A1	EADPTGHSY	Melanoma, other tumors [1]
MAGE-1	Cw*1601	SAYGEPRKL	Melanoma, other tumors [1]

MAGE-3	A1	EVDPIGHLY	Melanoma, other tumors [2]
MAGE-3	A2	FLWGPRALV	Melanoma, other tumors [2]
BAGE	Cw*1601	AARAVFLAL	Melanoma, other tumors [3]
GAGE-1,2	Cw6	YRPRPRRY	Melanoma, other tumors 4
HER-2/neu	A2	KIFGSLAFL, VMAGVGSPYV	Ovarian Cancer
HER-2/neu	A2	IISAVVGIL	Ovarian Cancer, NSCLC
CEA	A2	YLSGANLNL	Colon Cancer
p15	A24	(E)AYGLDFYIL	Melanoma and normal tissues
43kD protein	A2	QDLTMKYQIF	Melanoma
MUM-1 gene product mutated across intron/exon junction	B*4402	EEKLIVVLF [5]	Melanoma
mutated beta-catenin	A24	SYLDSGIHF [6]	Melanoma

[1] MAGE-1: expressed in Melanoma (36%), Bladder CA (19%), Breast CA (18%), Head & neck CA (25%), Non-small cell lung CA (NSCLC, 34%), Sarcomas (11%), Prostate CA (15%) [50]

[2] MAGE-3: expressed in Melanoma (65%), Bladder CA (34%),

Breast CA (11%), Head & neck CA (48%), Non-small cell lung CA (NSCLC, 31%), Sarcomas (11%), Prostate CA (15%) [50]

[3] BAGE: expressed in Melanoma (22%), Bladder CA (15%), Breast CA (10%), Head and neck CA (<10%), NSCLC (<10%) [50]

[4] GAGE-1, -2: expressed in Melanoma (24%), Bladder CA (12%), Breast CA (9%), Head & neck CA (19%), NSCLC (19%), Sarcomas (25%), Prostate cancers (10%) [50]

[5] Isoleucine (I) at position 5 is the result of mutation. The wild type sequence si EEKLSVVLF.

[6] Phenylalanine (F) at pos. 9 is the result of mutation. The wild type sequence is SYLDSGIHS.

If it is desirable to present more than one CTL epitope, rather than presenting all of the epitopes on a single immunogen, they may be presented on two or more different immunogens. These may be administered separately, or as part of a mixture, e.g., a mixture of epitopic peptides.

Mode of Production

The peptide portion of the immunogens of the present invention may be produced by any conventional technique, including

(a) nonbiological synthesis by sequential coupling of component amino acids,

(b) production by recombinant DNA techniques in a suitable host cell, and

(c) chemical or enzymatic modification of a sequence made by (a) or (b) above.

Gene Expression. The peptides disclosed herein may be produced, recombinantly, in a suitable host, such as bacteria from the genera Bacillus, Escherichia, Salmonella, Erwinia, and yeasts from the genera Hansenula, Kluyveromyces, Pichia, Rhinosporidium, Saccharomyces, and Schizosaccharomyces, or cultured mammalian cells such as COS-1. The more preferred hosts are microorganisms of the species *Pichia pastoris*, *Bacillus subtilis*, *Bacillus brevis*, *Saccharomyces cerevisiae*, *Escherichia coli* and *Yarrowia lipolytica*. Any promoter, regulatable or constitutive, which is functional in the host may be used to control gene expression.

It has been found that peptide fragments from the protein pMEL17 reconstitute HLA A2.1 and A3 epitopes. The pMEL17 gene is a single-stranded cDNA reading 5' to 3'. The gene encoding for pMEL17, is:

GGAAGAACAC AATGGATCTG GTGCTAAAAA GATGCCTTCT TCATTTGGCT
GTGATAGGTG CTTTGCTGGC TGTGGGGGCT ACAAAAGTAC CCAGAAACCA
GGACTGGCTT GGTGTCTCAA GGCAACTCAG AACCAAAGCC TGGAACAGGC
AGCTGTATCC AGAGTGGACA GAAGCCCAGA GACTTGACTG CTGGAGAGGT
GGTCAAGTGT CCCTCAAGGT CAGTAATGAT GGGCCTACAC TGATTGGTGC
AAATGCCTCC TTCTCTATTG CCTTGAACTT CCCTGGAAGC CAAAAGGTAT
TGCCAGATGG GCAGGTTATC TGGGTCAACA ATACCATCAT CAATGGGAGC
CAGGTGTGGG GAGGACAGCC AGTGTATCCC CAGGAAACTG ACGATGCCTG
CATCTTCCCT GATGGTGGAC CTTGCCCATC TGGCTCTTGG TCTCAGAAGA
GAAGCTTTGT TTATGTCTGG AAGACCTGGG GCCAATACTG GCAAGTTCTA
GGGGGCCCAG TGTCTGGGCT GAGCATTGGG ACAGGCAGGG CAATGCTGGG
CACACACACC ATGGAAGTGA CTGTCTACCA TCGCCGGGGA TCCCGGAGCT
ATGTGCCTCT TGCTCATTCC AGCTCAGCCT TCACCATTAC TGACCAGGTG
CCTTTCTCCG TGAGCGTGTC CCAGTTGCGG GCCTTGGATG GAGGGAACAA
GCACTTCCTG AGAAATCAGC CTCTGACCTT TGCCCTCCAG CTCCATGACC
CTAGTGGCTA TCTGGCTGAA GCTGACCTCT CCTACACCTG GGACTTTGGA
GACAGTAGTG GAACCCTGAT CTCTCGGGCA CCTGTGGTCA CTCATACTTA
CCTGGAGCCT GGCCCAGTCA CTGCCCAGGT GGTCCTGCAG GCTGCCATTC
CTCTCACCTC CTGTGGCTCC TCCCCAGTTC CAGGCACCAC AGATGGGCAC
AGGCCAACTG CAGAGGCCCC TAACACCACA GCTGGCCAAG TGCCTACTAC
AGAAGTTGTG GGTACTACAC CTGGTCAGGC GCCAACTGCA GAGCCCTCTG
GAACCACATC TGTGCAGGTG CCAACCACTG AAGTCATAAG CACTGCACCT
GTGCAGATGC CAACTGCAGA GAGCACAGGT ATGACACCTG AGAAGGTGCC
AGTTTCAGAG GTCATGGGTA CCACACTGGC AGAGATGTCA ACTCCAGAGG
CTACAGGTAT GACACCTGCA GAGGTATCAA TTGTGGTGCT TTCTGGAACC
ACAGCTGCAC AGGTAACAAC TACAGAGTGG GTGGAGACCA CAGCTAGAGA
GCTACCTATC CCTGAGCCTG AAGGTCCAGA TGCCAGCTCA ATCATGTCTA
CGGAAAGTAT TACAGGTTCC CTGGGCCCCC TGCTGGATGG TACAGCCACC
TTAAGGCTGG TGAAGAGACA AGTCCCCCTG GATTGTGTTC TGTATCGATA
TGGTTCCTTT TCCGTCACCC TGGACATTGT CCAGGGTATT GAAAGTGCCG
AGATCCTGCA GGCTGTGCCG TCCGGTGAGG GGGATGCATT TGAGCTGACT
GTGTCCTGCC AAGGCGGGCT GCCCAAGGAA GCCTGCATGG AGATCTCATC
GCCAGGGTGC CAGCCCCCTG CCCAGCGGCT GTGCCAGCCT GTGCTACCCA
GCCCAGCCTG CCAGCTGGTT CTGCACCAGA TACTGAAGGG TGGCTCGGGG

ACATACTGCC TCAATGTGTC TCTGGCTGAT ACCAACAGCC TGGCAGTGGT
CAGCACCCAG CTTATCATGC CTGTGCCTGG GATTCTTCTC ACAGGTCAAG
AAGCAGGCCT TGGGCAGGTT CGGCTGATCG TGGGCATCTT GCTGGTGTTG
ATGGCTGTGG TCCTTGCATC TCTGATATAT AGGCGCAGAC TTATGAAGCA
AGACTTCTCC GTACCCCAGT TGCCACATAG CAGCAGTCAC TGGCTGCGTC
TACCCCGCAT CTTCTGCTCT TGTCCCATTG GTGAGAATAG CCCCCTCCTC
AGTGGGCAGC AGGTCTGAGT ACTCTCATAT GATGCTGTGA TTTTCCTGGA
GTTGACAGAA ACACCTATAT TTCCCCCAGT CTTCCCTGGG AGACTACTAT
TAACTGAAAT AAATACTCAG AGCCTGAAAA A

The peptide 946L YLEPGPVTA reconstitutes an A2.1 epitope. Its native encoding gene sequence is TAC CTG GAG CCT GGC CAA GTC ACT GCC. Because this peptide has proven immunologic activity, it is ideal for specific immunization. Such immunization may be accomplished either directly, or by use of a vaccine consisting of virus (e.g., Vaccinia) encoding or HLA-A2 cells expressing a genetic sequence encoding this peptide. ·The peptide ALLAVGATK, which corresponds to residues 17-25 of pMel-17, reconstitutes an A3 epitope.

Also promising is the gene sequence encoding tyrosinase-related peptide 1030, YMDGTMSQV, natively encoded by TAT ATG GAT GGA ACA ATG TCC GAG GTA, which reconstitutes an A2-epitope, and that encoding KCDICTDEY, which reconstitutes an A1 epitope of tyrosinase.

The Genetic Code can readily be used to design a gene encoding an arbitrary amino acid sequence, such as that of the preferred HLA-A1 epitope, KCDICTDEY, or the preferred HLA-A3 epitope, ALLAVGATK. Preferably, where more than one codon could be used to encode a particular amino acid, consideration is given to the codon preferences of the intended host organism.

These sequences may be constructed in such a manner, including the appropriate expression systems for use in gene therapy procedures. Because several different nucleotide sequences may encode a single amino acid, alternate DNA sequences may also encode these peptides.

Standard reference works setting forth the general

principles of recombinant DNA technology include Watson, J.D., et al., Molecular Biology of the Gene, Volumes I and II, The Benjamin/Cummings Publishing Company, Inc., publisher, Menlo Park, CA (1987); Darnell, J.E., et al., Molecular Cell Biology, Scientific American Books, Inc., publisher, New York, N.Y. (1986); Lewin, B.M., Genes II, John Wiley & Sons, publishers, New York, N.Y. (1985); Old, R.W., et al., Principles of Gene Manipulation: An Introduction to Genetic Engineering, 2d edition, University of California Press, publisher, Berkeley, CA (1981); Sambrook, J., et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989); and Ausubel, et al., Current Protocols in Molecular Biology, Wiley Interscience, N.Y., (1987, 1992). These references are herein entirely incorporated by reference.

Chemical Peptide Synthesis. Chemical peptide synthesis is a rapidly evolving area in the art, and methods of solid phase peptide synthesis are well-described in the following references, hereby entirely incorporated by reference: (Merrifield, B., J. Amer. Chem. Soc. 85:2149-2154 (1963); Merrifield, B., Science 232:341-347 (1986); Wade, J.D., et al., Biopolymers 25:S21-S37 (1986); Fields, G.B., Int. J. Polypeptide Prot. Res. 35:161 (1990); MilliGen Report Nos. 2 and 2a, Millipore Corporation, Bedford, MA, 1987) Ausubel, et al, supra, and Sambrook, et al, supra.

In general, as is known in the art, such methods involve blocking or protecting reactive functional groups, such as free amino, carboxyl and thio groups. After polypeptide bond formation, the protective groups are removed (or de-protected). Thus, the addition of each amino acid residue requires several reaction steps for protecting and deprotecting. Current methods utilize solid phase synthesis, wherein the C-terminal amino acid is covalently linked to an insoluble resin particle large enough to be separated from the fluid phase by filtration. Thus, reactants are removed by washing the resin particles with appropriate solvents using an automated programmed machine. The completed polypeptide chain is cleaved from the resin by a reaction which does not

affect polypeptide bonds.

In the more classical method, known as the "tBoc method," the amino group of the amino acid being added to the resin-bound C-terminal amino acid is blocked with tert-butyloxycarbonyl chloride (tBoc). This protected amino acid is reacted with the bound amino acid in the presence of the condensing agent dicyclohexylcarbodiimide, allowing its carboxyl group to form a polypeptide bond the free amino group of the bound amino acid. The amino-blocking group is then removed by acidification with trifluoroacetic acid (TFA); it subsequently decomposes into gaseous carbon dioxide and isobutylene. These steps are repeated cyclically for each additional amino acid residue. A more vigorous treatment with hydrogen fluoride (HF) or trifluoro-methanesulfonyl derivatives is common at the end of the synthesis to cleave the benzyl-derived side chain protecting groups and the polypeptide-resin bond.

More recently, the preferred "Fmoc" technique has been introduced as an alternative synthetic approach, offering milder reaction conditions, simpler activation procedures and compatibility with continuous flow techniques. This method was used, e.g., to prepare the peptide sequences disclosed in the present application. Here, the α-amino group is protected by the base labile 9-fluorenylmethoxycarbonyl (Fmoc) group. The benzyl side chain protecting groups are replaced by the more acid labile t-butyl derivatives. Repetitive acid treatments are replaced by deprotection with mild base solutions, e.g., 20% piperidine in dimethyl-formamide (DMF), and the final HF cleavage treatment is eliminated. A TFA solution is used instead to cleave side chain protecting groups and the peptide resin linkage simultaneously.

At least three different peptide-resin linkage agents can be used: substituted benzyl alcohol derivatives that can be cleaved with 95% TFA to produce a peptide acid, methanolic ammonia to produce a peptide amide, or 1% TFA to produce a protected peptide which can then be used in fragment condensation procedures, as described by Atherton, E., et

al., J. Chem. Soc. Perkin Trans. 1:538-546 (1981) and Sheppard, R.C., et al., Int. J. Polypeptide Prot. Res. 20:451-454 (1982). Furthermore, highly reactive Fmoc amino acids are available as pentafluorophenyl esters or dihydro-oxobenzotriazine esters derivatives, saving the step of activation used in the tBoc method.

Pharmaceutical Methods and Preparations

The preferred animal subject of the present invention is a primate mammal. By the term "mammal" is meant an individual belonging to the class Mammalia, which, of course, includes humans. The invention is particularly useful in the treatment of human subjects, although it is intended for veterinary uses as well. By the term "non-human primate" is intended any member of the suborder Anthropoidea except for the family Hominidae. Such non-human primates include the superfamily Ceboidea, family Cebidae (the New World monkeys including the capuchins, howlers, spider monkeys and squirrel monkeys) and family Callithricidae (including the marmosets); the superfamily Cercopithecoidea, family Cercopithecidae (including the macaques, mandrills, baboons, proboscis monkeys, mona monkeys, and the sacred hunaman monkeys of India); and superfamily Hominoidae, family Pongidae (including gibbons, orangutans, gorillas, and chimpanzees). The rhesus monkey is one member of the macaques.

The term "protection", as used herein, is intended to include "prevention," "suppression" and "treatment." "Prevention" involves administration of the protein prior to the induction of the disease. "Suppression" involves administration of the composition prior to the clinical appearance of the disease. "Treatment" involves administration of the protective composition after the appearance of the disease.

It will be understood that in human and veterinary medicine, it is not always possible to distinguish between "preventing" and "suppressing" since the ultimate inductive event or events may be unknown, latent, or the patient is not ascertained until well after the occurrence of the event or

events. Therefore, it is common to use the term "prophylaxis" as distinct from "treatment" to encompass both "preventing" and "suppressing" as defined herein. The term "protection," as used herein, is meant to include "prophylaxis." It should also be understood that to be useful, the protection provided need not be absolute, provided that it is sufficient to carry clinical value. An agent which provides protection to a lesser degree than do competitive agents may still be of value if the other agents are ineffective for a particular individual, if it can be used in combination with other agents to enhance the level of protection, or if it is safer than competitive agents.

The composition may be administered parentally or orally, and, if parentally, either systemically or topically. Parenteral routes include subcutaneous, intravenous intradermal, intramuscular, intraperitoneal, intranasal, transdermal, or buccal routes. One or more such routes may be employed. Parenteral administration can be, e.g., by bolus injection or by gradual perfusion over time. Alternatively, or concurrently, administration may be by the oral route. The immunization is preferably accomplished initially by intramuscular injection followed by intradermal injection, although any combination of intradermal and intramuscular injections may be used.

It is understood that the suitable dosage of a immunogen of the present invention will be dependent upon the age, sex, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired. However, the most preferred dosage can be tailored to the individual subject, as is understood and determinable by one of skill in the art, without undue experimentation. This will typically involve adjustment of a standard dose, e.g., reduction of the dose if the patient has a low body weight.

Prior to use in humans, a drug will first be evaluated for safety and efficacy in laboratory animals. In human clinical studies, one would begin with a dose expected to be

safe in humans, based on the preclinical data for the drug in question, and on customary doses for analogous drugs (if any). If this dose is effective, the dosage may be decreased, to determine the minimum effective dose, if desired. If this dose is ineffective, it will be cautiously increased, with the patients monitored for signs of side effects. See, e.g., Berkow, et al., eds., The Merck Manual, 15th edition, Merck and Co., Rahway, N.J., 1987; Goodman, et al., eds., Goodman and Gilman's The Pharmacological Basis of Therapeutics, 8th edition, Pergamon Press, Inc., Elmsford, N.Y., (1990); Avery's Drug Treatment: Principles and Practice of Clinical Pharmacology and Therapeutics, 3rd edition, ADIS Press, LTD., Williams and Wilkins, Baltimore, MD. (1987), Ebadi, Pharmacology, Little, Brown and Co., Boston, (1985), which references and references cited therein, are entirely incorporated herein by reference.

The total dose required for each treatment may be administered in multiple doses (which may be the same or different) or in a single dose, according to an immunization schedule, which may be predetermined or ad hoc. The schedule is selected so as to be immunologically effective, i.e., so as to be sufficient to elicit an effective CTL response to the antigen and thereby, possibly in conjunction with other agents, to provide protection. The doses adequate to accomplish this are defined as "therapeutically effective doses." (Note that a schedule may be immunologically effective even though an individual dose, if administered by itself, would not be effective, and the meaning of "therapeutically effective dose" is best interpreted in the context of the immunization schedule.) Amounts effective for this use will depend on, e.g., the peptide composition, the manner of administration, the stage and severity of the disease being treated, the weight and general state of health of the patient, and the judgment of the prescribing physician, but generally range for the initial immunization (that is for therapeutic or prophylactic administration) from about 1.0 μg to about 5000 μg of peptide for a 70 kg patient, followed by boosting dosages of from about 1.0 μg to about

1000 μg of peptide pursuant to a boosting regimen over weeks to months depending upon the patient's response and condition by measuring specific CTL activity in the patient's blood. It must be kept in mind that the peptides and compositions of the present invention may generally be employed in serious disease states, that is, life-threatening or potentially life threatening situations. In such cases, in view of the minimization of extraneous substances and the relative nontoxic nature of the peptides, it is possible and may be felt desirable by the treating physician to administer substantial excesses of these peptide compositions.

The doses may be given at any intervals which are effective. If the interval is too short, immunoparalysis or other adverse effects can occur. If the interval is too long, immunity may suffer. The optimum interval may be longer if the individual doses are larger. Typical intervals are 1 week, 2 weeks, 4 weeks (or one month), 6 weeks, 8 weeks (or two months) and one year. The appropriateness of administering additional doses, and of increasing or decreasing the interval, may be reevaluated on a continuing basis, in view of the patient's immunocompetence (e.g., the level of antibodies to melanoma-associated antigens).

The concentration of CTL stimulatory peptides of the invention in the pharmaceutical formulations can vary widely, i.e., from less than about 0.1%, usually at or at least about 2% to as much as 20% to 50% or more by weight, and will be selected primarily by fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected.

In one embodiment, the immunogen is dissolved or suspended in an aqueous carrier. A variety of aqueous carriers may be used, e.g., water, buffered water, 0.9% saline, 0.3% glycine, hyaluronic acid and the like. These compositions may be sterilized by conventional, well known sterilization techniques, or may be sterile filtered. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile solution prior to administration. The compositions

may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, etc.

The peptides of the invention may also be administered via liposomes, which serve to target the peptides to a particular tissue, as well as increase the half-life of the peptide composition. Liposomes include emulsions, foams, micelles, insoluble monolayers, liquid crystals, phospholipid dispersions, lamellar layers and the like. In these preparations the peptide to be delivered is incorporated as part of a liposome, alone or in conjunction with a molecule which binds to, e.g., a receptor prevalent among melanocytes or melanomas, or with other therapeutic or immunogenic compositions. Thus, liposomes filled with a desired peptide of the invention can be directed to the site of target cells, where the liposomes then deliver the selected therapeutic/immunogenic peptide compositions. Liposomes for use in the invention are formed from standard vesicle-forming lipids, which generally include neutral and negatively charged phospholipids and a sterol, such as cholesterol. The selection of lipids is generally guided by consideration of, e.g., liposome size, acid lability and stability of the liposomes in the blood stream. A variety of methods are available for preparing liposomes, as described in, e.g., Szoka et al., Ann. Rev. Biophys. Bioeng. 9:467 (1980), U.S. Patent Nos. 4,235,871, 4,501,728, 4,837,028, and 5,019369, incorporated herein by reference.

For targeting to the melanoma cells, a ligand to be incorporated into the liposome can include, e.g., antibodies or fragments thereof specific for cell surface determinants of the desired melanoma cells. A liposome suspension containing a peptide may be administered intravenously, locally, topically, etc. in a dose which varies according to, *inter alia*, the manner of administration, the peptide being delivered, and the stage of the disease being treated.

For solid compositions, conventional nontoxic solid carriers may be used which include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like. For oral administration, a pharmaceutically acceptable nontoxic composition is formed by incorporating any of the normally employed excipients, such as those carriers previously listed, and generally 10-95% of active ingredient, that is, one or more peptides of the invention, and more preferably at a concentration of 25%-75%.

For aerosol administration, the immunogenic peptides are preferably supplied in finely divided form along with a surfactant and propellant. Typical percentages of peptides are 0.01%-20% by weight, preferably 1%-10%. The surfactant must, of course, be nontoxic, and preferably soluble in the propellant. Representative of such agents are the esters or partial esters of fatty acids containing from 6 to 22 carbon atoms, such as caproic, octanoic, lauric, palmitic, stearic, linoleic, linolenic, olesteric and oleic acids with an aliphatic polyhydric alcohol or its cyclic anhydride. Mixed esters, such as mixed or natural glycerides may be employed. The surfactant may constitute 0.1%-20% by weight of the composition, preferably 0.25-5%. the balance of the composition is ordinarily propellant. A carrier can also be included, as desired, as with, e.g., lecithin for intranasal delivery.

In addition to the peptides or analogues of the invention, a pharmaceutical composition may contain suitable pharmaceutically acceptable carriers, such as excipients, carriers and/or auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically.

The appropriate dosage form will depend on the disease, the immunogen, and the mode of administration; possibilities include tablets, capsules, lozenges, dental pastes, suppositories, inhalants, solutions, ointments and parenteral depots. See, e.g., Berker, supra, Goodman, supra, Avery,

supra and Ebadi, supra, which are entirely incorporated herein by reference, including all references cited therein. However, it is expected that each vaccine preparation will include 1-100 μg of the peptide epitope.

The composition may also include an adjuvant. Typical adjuvants include proteins, peptides, carbohydrates, lipids and liposaccharides. An example of a currently popular adjuvant is DETOX (Ribi Immunochemicals)(muramyl dipeptide and cell wall fragments from Mycobacterium phlei). Other adjuvants include QS-21, Montanide ISA-21, incomplete Freund's adjuvant, aluminum phosphate, aluminum hydroxide, alum, DEAE-dextran, saponin, and mineral oil. Montanide ISA-51 is manufactured by Seppic, Inc. (75 Quai D'Orsay, 75321, Paris, France). Its composition is manide oleate in mineral oil solution.

QS-21 is manufactured by Cambridge Biotech (365 Plantation Street, Worcester, MA 01605-2376). It is a triterpene glycoside isolated from the bark of a South American tree (Quillaja saponaria). The tradename for QS-21 is Stimulon™. Its molecular formula is $C_{92}O_{46}H_{148}$, and its molecular weight is 1,990. Its complete chemical name is

3-O-β-D-galactopyranosyl-(1->2)-[β-D-xylopyranosyl-(1->3)]-β-D-glucuronpyranosyl-quillaic acid 28-O-β-D-apiofuranosyl-(1->3)-β-D-xylopyranosyl-(1->4)-α-L-rhamnopyranosyl-(1->2)-3-[5-O-α-L-arabinofuranosyl 3,5-dihydroxy-6-methyloctanoyl]-3,5-dihydroxy-6-methyloctanoyl]-β-D-fucopyranoside.

If desired, the adjuvant may be conjugated to the epitope and not simply a part of a mixture. See Deres, et al, Nature, 342:561-4 (1989).

The composition may also include an immunomodulator, especially cytokines such as IL-1, IL-2, IL-4, IL-6, IL-7, IL-12, Interferon-alpha, Interferon-gamma, Granulocyte Macrophage Colony Stimulating Factor (GMCSF), Tumor Necrosis Factor (TNF)-alpha, and TNF- beta.

The composition may also include antigen-presenting cells, such as dendritic cells or macrophages. Preferably, the APCs are harvested, e.g., from peripheral blood or bone

marrow, conjugated, covalently or noncovalently (e.g., by pulsing) to the immunogen, e.g., a peptide, and administered to the patient.

The composition may also include a molecule which activates or helps in activating CTLs, such as a CD-28 stimulatory molecule (e.g., B7.1, B7.2, or anti-CD28). If the molecule may be administered in place of the molecule itself.

CD80 (B7 BB1) is expressed on activated B cells and dendritic cells. It is a ligand for CD28 and CTLA-4. It has been found to represent two (partially homologous) proteins, B7-1 and B7-2. See Ramarathinam, et al. T cell costimulation by B7/BB1 induces CD8 T-cell-dependent tumor rejection: an important role of B7/BB1 in the induction, recruitment, and effector function of antitumor T cells. J.Exp. Med. 1994: 1790: 1205-1214; Freeman et al. Cloning of B7-2: a CTLA-4 counter-receptor that costimulates human T cell proliferation. Science 1993, 262: 909-911; Li et al. Costimulation of tumor-reactive CD4+ and CD8+ T lymphocytes by B7, a natural ligand for CD28, can be used to treat established mouse melanoma. J. Immunol. 1994, 153: 421-428; Hodge et al. Admixture of a recombinant vaccinia virus containing the gene for the costimulator molecule B7 and a recombinant vaccinia virus containing a tumor-associated antigen gene results in enhanced specific T-cell responses antitumor immunity. Cancer Res. 1995, 55: 3598-3603.

A pharmaceutical composition according to the present invention may further comprise at least one cancer chemotherapeutic compound, such as one selected from the group consisting of an anti-metabolite, a bleomycin peptide antibiotic, a podophyllin alkaloid, a *Vinca* alkaloid, an alkylating agent, an antibiotic, cisplatin, or a nitrosourea. A pharmaceutical composition according to the present invention may further or additionally comprise at least one viral chemotherapeutic compound selected from gamma globulin, amantadine, guanidine, hydroxybenzimidazole, interferon-α, interferon-β, interferon-γ, thiosemicarbarzones, methisazone, rifampin, ribvirin, a pyrimidine analog, a purine analog,

foscarnet, phosphonoacetic acid, acyclovir, dideoxynucleosides, or ganciclovir. See, e.g., Katzung, *supra*, and the references cited therein on pages 798-800 and 680-681, respectively, which references are herein entirely incorporated by reference.

As an alternative to a pharmaceutical composition comprising the immunogen of the present invention, per se, the pharmaceutical composition may instead comprise a vector comprising an expressible gene encoding such an immunogen. The pharmaceutical composition and method would then be chosen so that the vector was delivered to suitable cells of the subject, so that the gene would be expressed and the immunogen produced in such a manner as to elicit an immune response. A preferred vector would be a Vaccinia virus, such as a construct containing a minigene encoding the peptide 946L (YLEPGPVTA), 946I ((YIEPGPVTA), 1030 (SEQ. ID. NO.: 9) or ALLAVGATK. A gene encoding the protein pMel-17 is also of some interest. In the case of genes encoding naturally occurring proteins, or peptide fragments thereof, one may, but need not, use the DNA sequence which encodes the proteins or peptides in nature. A preferred route for immunization would be scarification. A preferred immunization protocol would be 10E6 to 10E8 pfu/dose in the initial injection, followed up with boosters at 1,3 and 12 months. The boosters could be the previously described immunogen-containing composition.

In the case of genes encoding naturally occurring proteins, or peptide fragments thereof, one may, but need not, use the DNA sequence which encodes the proteins or peptides in nature.

Recombinant vaccinia virus constructs have been used for immunization against hepatitis B (Moss, et al., Nature, 311, 67, 1984), herpes simplex virus (Wacchsman, et al., Biosci. Rep. 8, 323; 334, 1988), parainfluenza type 3 (Spriggs, et al., J. Virol., 62, 1293, 1988), and Lassa fever virus (Fisher-Hoch, et al., Proc. Natl. Acad. Sci. USA, 86, 317, 1989). Vaccinia virus constructs comprising gene for cancer-associated antigens have also been prepared (Lathe, et al.,

Nature, 326, 878, 1987; Bernards, et al., Proc. Natl. Acad. Sci. USA, 84, 6854, 1987; Estin, et al., Proc. Natl. Acad. Sci. USA, 85, 1052, 1988).

Alternatively or additionally, the composition may comprise melanoma-specific CTL. Antigenic peptides may be used to elicit CTL ex vivo. Ex vivo CTL responses to a melanoma antigen are induced by incubating in tissue culture the patient's CTL precursor cells (CTLp) together with a source of antigen-presenting cells (APC) and the appropriate immunogenic peptide. After an appropriate incubation time (typically 1-8 weeks), in which the CTLp are activated and mature and expand into effector CTL, the cells are infused back into the patient, where they will destroy their specific target cell. In order to optimize the *in vitro* conditions for the generation of specific cytotoxic T cells, the culture of stimulator cells may be maintained in an appropriate serum-free medium.

Prior to incubation of the stimulator cells with the cells to be activated, e.g., precursor CD8+ cells, an amount of antigenic peptide is added to the stimulator cell culture, of sufficient quantity to become loaded onto the human Class I molecules to be expressed on the surface of the stimulator cells. In the present invention, a sufficient amount of peptide is an amount that will allow about 200, and preferably 200 or more, human Class I MHC molecules loaded with peptide to be expressed on the surface of each stimulator cell. Preferably, the stimulator cells are incubated with at least 1 mg/ml, more preferably >20μg/ml peptide.

Resting or precursor CD8+ cells are then incubated in culture with the appropriate stimulator cells for a time period sufficient to activate the CD8+ cells. Preferably, the CD8+ cells are activated in an antigen-specific manner. The ratio of resting or precursor CD8+ (effector) cells to stimulator cells may vary from individual to individual and may further depend upon variables such as the amenability of an individual's lymphocytes to culturing conditions and the nature and severity of the disease condition or other

condition for which the within-described treatment modality is used. Preferably, however, the lymphocyte:stimulator cell ratio is in the range of about 1:5 to 20:1, more preferably 3:1 to 5:1. The effector/stimulator culture may be maintained for as long a time as is necessary to stimulate a therapeutically useful or effective number of CD8+ cells.

The induction of CTL *in vitro* requires the specific recognition of peptides that are bound to allele specific MHC class I molecules on APC. The number of specific MHC/peptide complexes per APC is crucial for the stimulation of CTL, particularly in primary immune responses. While small amounts of peptide/MHC complexes per cell are sufficient to render a cell susceptible to lysis by CTL, or to stimulate a secondary CTL response, the successful activation of a CTL precursor (pCTL) during primary response requires a significantly higher number of MHC/peptide complexes. Peptide loading of empty major histocompatability complex molecules on cells allows the induction of primary cytotoxic T lymphocyte responses.

Often it is useful, in the generation of peptide-specific CTL, to stimulate with mutant cell lines that have some empty MHC molecules. An exmample is the human lymphoid cell line, T2. However, mutant cell lines expressing every MHC molecule are not yet available. Thus, in some cases, it may be useful to strip endogenous MHC-associated peptides from the surface of APC, followed by loading the resulting empty MHC molecules with the immunogenic peptides of interest. The use of non-transformed (non-tumorigenic), non-infected cells, and preferably, autologous cells of patients as APC is desirable for the design of CTL induction protocols directed towards development of *ex vivo* CTL therapies. This application discloses methods for stripping the endogenous MHC-associated peptides from the surface of APC followed by the loading of desired peptides.

A stable MHC class I molecule is a trimeric complex formed of the following elements: 1) a peptide usually of 8 - 10 residues, 2) a transmembrane heavy polymorphic protein chain which bears the peptide-binding site in its $\alpha 1$ and $\alpha 2$

domains, and 3) a non-covalently associated non-polymorphic light chain, $\beta_2$microglobulin. Removing the bound peptides and/or dissociating the $\beta_2$microglobulin from the complex renders the MHC class I molecules nonfunctional and unstable, resulting in rapid degradation. All MHC class I molecules isolated from PBMCs have endogenous peptides bound to them. Therefore, the first step is to remove all endogenous peptides bound to MHC class I molecules on the APC without causing their degradation before exogenous peptides can be added to them.

Two possible ways to free up MHC class I molecules of bound peptides include the culture temperature from 37°C to 26°C overnight to destabilize $\beta_2$microglobulin and stripping the endogenous peptides from the cell using a mild acid treatment. The methods release previously bound peptides into the extracellular environment allowing new exogenous peptides to bind to the empty class I molecules. The cold-temperature incubation method enables exogenous peptides to bind efficiently to the MHC complex, but requires an overnight incubation at 26°C which may slow the cell's metabolic rate. It is also likely that cells not actively synthesizing MHC molecules (e.g., resting PBMC) would not produce high amounts of empty surface MHC molecules by the cold temperature procedure.

Harsh acid stripping involves extraction of the peptides with trifluoroacetic acid, pH 2, or acid denaturation of the immunoaffinity purified class I-peptide complexes. These methods are not feasible for CTL induction, since it is important to remove the endogenous peptides while preserving APC viability and an optimal metabolic state which is critical for antigen presentation. Mild acid solutions of pH 3 such as glycine or citrate-phosphate buffers have been used to identify endogenous peptides and to identify tumor associated T cell epitopes. The treatment is especially effective, in that only the MHC class I molecules are destabilized (and associated peptides released), while other surface antigens remain intact, including MHC class II molecules. Most importantly, treatment of cells with the

mild acid solutions do not affect the cell's viability or metabolic state. The mild acid treatment is rapid since the stripping of the endogenous peptides occurs in two minutes at 4°C and the APC is ready to perform its function after the appropriate peptides are loaded. The technique is utilized herein to make peptide-specific APCs for the generation of primary antigen-specific CTL. The resulting APC are efficient in inducing peptide-specific CD8+ CTL.

Activated CD8+ cells may be effectively separated from the stimulator cells using one of a variety of known methods. For example, monoclonal antibodies specific for the stimulator cells, for the peptides loaded onto the stimulator cells, or for the CD8+ cells (or a segment thereof) may be utilized to bind their appropriate complementary ligand. Antibody-tagged molecules may then be extracted from the stimulator-effector cell admixture via appropriate means, e.g., via well-known immunoprecipitation or immunoassay methods.

Effective, cytotoxic amounts of the activated CD8+ cells can vary between *in vitro* and *in vivo* uses, as well as with the amount and type of cells that are the ultimate target of these killer cells. The amount will also vary depending on the condition of the patient and should be determined via consideration of all appropriate factors by the practitioner. Preferably, however, about 1 X 10^6 to about 1 X 10^{12}, more preferably about 1 X 10^8 to about 1 X 10^{11}, and even more preferably, about 1 X 10^9 to about 1 X 10^{10} activated CD8+ cells are utilized for adult humans, compared to about 5 X 10^6 - 5 X 10^7 cells are used in mice.

Preferably, as discussed above, the activated CD8+ cells are harvested from the cell culture prior to administration of the CD8+ cells to the individual being treated. It is important to note, however, that unlike other present and proposed treatment modalities, the present method preferably uses a cell culture system that is not tumorigenic. Therefore, if complete separation of stimulator cells and activated CD8+ cells is not achieved, there is no inherent danger known to be associated with the administration of a

small number of stimulator cells, whereas administration of mammalian tumor-promoting cells may be extremely hazardous.

Methods of re-introducing cellular components are known in the art and include procedures such as those exemplified in U.S. Patent No. 4,844,893 to Honsik, et al. and U.S. Patent No. 4,690,915 to Rosenberg. For example, administration of activated CD8+ cells via intravenous infusion is appropriate.

Adoptive transfer of melanoma-specific CTL has been accompanied by tumor shrinkage in a large minority of patients with advanced melanoma and by disappearance of all detectable tumor in a smaller proportion of patients. (Rosenberg et al, NEM 319: 1676-1680, 1988) and in animal studies appears to be particularly promising for the treatment of solid tumors (Rosenberg SA et al. Science 233:1318-1321). One of the problems with existing methods for CTL generation is that they require the resection of large metastic tumor deposits to initiate the process. If the requirement for available autologous tumor could be circumvented, then patients with no measurable disease but a high risk of recurrence (eg, melanoma patients with primary tumors greater than 4 mm thick or with microscopic tumor metastatic to regional nodes) could be treated with adoptive therapy even if their tumor were removed and fixed in formalin and no other gross tumor was evident. These patients have a very high likelihood of harboring micrometastic disease for which no other effective therapy is now available; so most will die of the melanoma. It is possible that the presence of bulky tumor suppresses the autologous immune response; so treatment of patients without bulky disease would be an attractive goal. Especially in murine systems, CTL have been generated and maintained by stimulation with cells to which the peptide epitope has been bound. We propose that, e.g., HLA-A2.1+ or HLA-A3+ cells (autologous B cells, macrophages, or dendritic cells, ideally), would be pulsed in vitro with peptide (e.g., peptide 946, YXEPGPVTA) and used as in vitro simulators for autologous lymph node cells or peripheral blood lymphocytes.

The patients could be pre-stimulated with a peptide vaccine prior lymphocyte harvest if the existing response was inadequate. Lymphocytes stimulated with peptide in vitro could then be expanded to 10^{10} or 10^{11} cells, then re-infused into the patients in a manner analogous to that used for LAK cell therapy. It is expected that the adoptively transferred CTL would survive best with IL-2 infusion at low to intermediate doses, and that putative inhibitors of Tc suppression (eg: cyclophosphamide) may be employed also, prior to the infusions of CTL.

Clinical studies with adoptive immunotherapy using A2-restricted tumor infiltrating lymphocytes (TIL) have shown a strong correlation between Pmel-17/gp100 reactivity and positive clinical responses of patients treated with those TIL. Kawakami, et al., J. Immunol., 154:3961-8 (1995).

Melanoma-Specific Diagnostic Agents

A melanoma-specific diagnostic agent is (1) a molecule which is or which comprises a melanoma-specific epitope as previously defined, and which is labeled, immobilized, or otherwise rendered suitable for diagnostic use, or (2) an antibody which specifically binds such a melanoma-specific epitope, and which is labeled, immobilized, or otherwise rendered suitable for diagnostic use, or (3) a T-cell line (e.g., murine or human), which specifically recognizes a melanoma-specific epitope.

Diagnostic Uses and Compositions

The relationship between the host's immune response and his or her tumor is poorly understood. Better understanding of that response depends on evaluation of the specific responses against individual epitopes, such as the 946 peptide. If patients do have an immune response to 946 naturally, then evaluation and quantitation of that by precursor frequency analysis of the CTL in the patient's blood pool may permit some assessment of the protection that person's immune system is providing. As new therapies become available for melanoma, it may be useful to screen patients

for the presence of the 946 peptide on their tumor and the presence of CTL in their blood pool with specificity for the 946 peptide on HLA-A2. In like manner one may screen for ALLAVGATK peptides on the tumor and for anti-ALLAVGATK CTLs in the blood of A3+ patients. These findings may determine whether further augmentation of the immune response is indicated or whether other, non-immunologic, therapy should be employed. A parallel to this is the determination on breast cancers of the presence of estrogen and progesterone receptors before considering hormonal therapy or chemotherapy.

Thus, the peptides of the present invention may be used to screen a sample for the presence of an antigen with the same epitope, or with a different but cross-reactive epitope, or for the presence of CTLs which specifically recognize the corresponding epitopes. The sample will normally be a biological fluid, such as blood, urine, lymphatic fluid, amniotic fluid, semen, saliva, tears, milk, or cerebrospinal fluid, or a fraction or derivative thereof, or a biological tissue, in the form of, e.g., a tissue section or homogenate. The preferred sample is blood, or a fraction or derivative thereof.

Assays may be divided into two basic types, heterogeneous and homogeneous. In heterogeneous assays, the interaction between the affinity molecule and the analyte does not affect the label, hence, to determine the amount or presence of analyte, bound label must be separated from free label. In homogeneous assays, the interaction does affect the activity of the label, and therefore analyte levels can be deduced without the need for a separation step.

Assays may also be divided into competitive and non-competitive formats. In the competitive format, the analyte competes with a labeled analyte analogue for binding to a binding partner. In a common noncompetitive format called a sandwich assay, the analyte is first bound by a capture reagent, and then by a tag reagent.

In order to detect the presence, or measure the amount, of an analyte, the assay must provide for a signal producing

system (SPS) in which there is a detectable difference in the signal produced, depending on whether the analyte is present or absent (or, in a quantitative assay, on the amount of the analyte). The detectable signal may be one which is visually detectable, or one detectable only with instruments. Possible signals include production of colored or luminescent products, alteration of the characteristics (including amplitude or polarization) of absorption or emission of radiation by an assay component or product, and precipitation or agglutination of a component or product. The term "signal" is intended to include the discontinuance of an existing signal, or a change in the rate of change of an observable parameter, rather than a change in its absolute value. The signal may be monitored manually or automatically.

The component of the signal producing system which is most intimately associated with the diagnostic reagent is called the "label". A label may be, e.g., a radioisotope, a fluorophore, an enzyme, a co-enzyme, an enzyme substrate, an electron-dense compound, an agglutinable particle.

The radioactive isotope can be detected by such means as the use of a gamma counter or a scintillation counter or by autoradiography. Isotopes which are particularly useful for the purpose of the present invention are ^{3}H, ^{125}I, ^{131}I, ^{35}S, ^{14}C, and, preferably, ^{125}I.

It is also possible to label a compound with a fluorescent compound. When the fluorescently labeled antibody is exposed to light of the proper wave length, its presence can then be detected due to fluorescence. Among the most commonly used fluorescent labelling compounds are fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine.

Alternatively, fluorescence-emitting metals such as ^{125}Eu, or others of the lanthanide series, may be attached to the binding protein using such metal chelating groups as diethylenetriaminepentaacetic acid (DTPA) and ethylenediaminetetraacetic acid (EDTA).

The peptides also can be detectably labeled by coupling

to a chemiluminescent compound. The presence of the chemiluminescently labeled antibody is then determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of particularly useful chemiluminescent labeling compounds are luminol, isolumino, theromatic acridinium ester, imidazole, acridinium salt and oxalate ester.

Likewise, a bioluminescent compound may be used to label the peptides. Bioluminescence is a type of chemiluminescence found in biological systems in which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence of luminescence. Important bioluminescent compounds for purposes of labeling are luciferin, luciferase and aequorin.

Enzyme labels, such as horseradish peroxidase, alkaline phosphatase, malate dehydrogenase, staphylococcal nuclease, δ-V-steroid isomerase, yeast alcohol dehydrogenase, α-glycero phosphate dehydrogenase, triose phosphate isomerase, asparaginase, glucose oxidase, β-galactosidase, ribonuclease, glucose-6-phosphate dehydrogenase, glucoamylase and acetylcholine esterase, are preferred. When an enzyme label is used, the signal producing system must also include a substrate for the enzyme. If the enzymatic reaction product is not itself detectable, the SPS will include one or more additional reactants so that a detectable product appears.

A label may be conjugated, directly or indirectly (e.g., through a labeled antibody), covalently (e.g., with SPDP) or noncovalently, to the peptide, to produce a diagnostic reagent. Similarly, the peptide may be conjugated to a solid phase support to form a solid phase ("capture") diagnostic reagent. Suitable supports include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, agaroses, and magnetite. The nature of the carrier can be either soluble to some extent or insoluble for the purposes of the present invention. The support material may have virtually any possible structural configuration so long as the coupled

molecule is capable of binding to its target. Thus the support configuration may be spherical, as in a bead, or cylindrical, as in the inside surface of a test tube, or the external surface of a rod. Alternatively, the surface may be flat such as a sheet, test strip, etc.

Additionally, the peptides may be used as a diagnostic tool to evaluate whether other immunotherapeutic treatments (tumor vaccines of any kind, adoptive transfer of CTL, etc) are having a beneficial effect.

Also the peptides 946L (YLEPGPVTA) and 946I (YIEPGPVTA) have low to intermediate affinity for the HLA-A2.1 molecule. This is illustrated in Figure 11. For this reason, they will be useful as control peptides for the evaluation of candidate peptide/MHC binding affinity. Because they represent a low affinity range, they can be used in laboratory studies on binding affinity of other peptides. This methodology, in a preferred embodiment, would likely include: binding the peptide to T2 cells, then evaluating lysis of the T2 cells by any of various standard methods, such as a proliferative response of the CTL, or cytokine release by the CTL exposed to the T2 cells+ peptide.

Fibroblasts GM126 were obtained from the National Institute of General Medical Sciences Human Genetic Mutant Cell Repository, Bethesa, MD. Melanoma lines DM6, DM13, DM14, and DM93 were the gift of Drs. Hilliard F. Siegler and Timothy L. Darrow. VMM1 and VMM5 are melanoma cell lines established from metastatic melanoma resected from patients at the University of Virginia (Charlottesville, VA). VBT2 (squamous cell lung carcinoma), VAO1 (adenocarcinoma of the ovary), and VAB5 (adenocarcinoma of the breast) are cell lines also established at this institution. JY, MICH, MWF, 23.1, RPMI 1788, and Herluff are EBV-transformed B lymphoblastoid lines. K562 is a NK-sensitive human erythroleukemia line. The cell line T2 is derived from the fusion of a T cell line, CEM, and a human B cell mutant, LCL 721.174. This cell line expresses HLA-A2.1 molecules but has an Ag-processing defect that is associated with enhanced presentation of exogenous peptides.

HLA Types of Cell Lines

The HLA types of several cell lines are listed in Table 1.

TABLE 1
Human cell lines used in this study: HLA types and susceptibility to lysis by VMM5 CTL[1a]

Cell Line (Ref.)	Cell Type[b]	HLA-A	HLA-B	HLA-C	HLA-DR	HLA-DQ	Lysis by VMM5 CTL[a]
DM6 (11)	Melanoma[f]	2.1[c]	12, 13 or 35	1, 2	6.10.(7)[d]	---[e]	++
DM13 (11)	Melanoma	2.1, 31	13, 18	ND	ND	ND	+++
DM14 (11)	Melanoma	11, 28	5, 8	2, 4	---	---	--
DM93 (11)	Melanoma	2.1, 33	8, 49, w6	ND	2, 4, 6[d]	3	++
SkMel24 (26)	Melanoma	1, 2.1	12, 14	---	---	---	--
HT144 (26)	Melanoma	1, 24	13, 15	3	4, 7	---	--
HT144 A2--03	Melanoma	1, 2.1, 24	13, 15	3	4, 7	---	+
VMM1	Melanoma	3, 26	51, w4, w6	ND	---	---	--
VMM5	Melanoma	2.1	39	ND	7, 11, 52, 53	2, 7	+++
VBT2	Lung CA	34, 68	35, (53?)	4?	---	---	--
VAO1	Ovarian CA	2	---	---	---	---	--
VAB5	Breast CA	2, 25	60, 62	3	---	---	--
MDAMB468 (26)	Breast CA	23, 30	27, 35	2, 4	---	---	--
CCL228 (26)	Colon CA	2.1	8, 17	---	---	---	--
143b (29)	Osteosarcoma	2.1	---	---	---	---	--
GM126 (29)	Fibroblasts	2.1	---	---	---	---	--
K562	Erythroleukemia	---	---	---	---	---	--
MICH (28)	EBV-B[g]	2.1, 32	15, 27	---	5, 5	---	--
RPMI-1788 (26)	EBV-B	2.1, 33	7, 14	---	---	---	--
JY (28)	EBV-B	2.1, 2.1	7, 7	---	4, 6	---	--
Herluff (27)	EBV-B	2.1, 2.1	12, 35	---	---	---	--
23.1 (28)	EBV-B	2, 2	27, 27	---	8, 8	---	--

REFERENCE EXAMPLE

The identification of melanoma-specific HLA-A2 epitopes of pMel-17 and tyrosinase is described in WO95/22561, incorporated by reference herein.

EXAMPLE IX

In the present example, we demonstrate that HLA-A3-restricted CTL recognize shared antigens on autologous and allogeneic melanoma cells, including an HLA-A3-restricted peptide derived from Pmel-17/gp100 and one or more peptides not yet identified, but apparently not derived from Pmel-17/gp100. These results support the use of Pmel-17/gp100-directed immunotherapy for patients who are HLA-A3$^+$, and suggest that HLA-A3, like HLA-A2, presents multiple shared melanoma antigens to HLA-A3 restricted CTL.

Materials and Methods

Cell lines and HLA typing: The human melanoma cell lines VMM1, VMM12, VMM18 and VMM34 were derived from patients at the University of Virginia (Charlottesville, VA). DM6 was provided by Drs. H.F. Seigler and T.L. Darrow at Duke University (Durham, NC). SkMel-2 was obtained from the American Type Culture Collection (ATCC, Bethesda, MD). Immunohistochemical staining of these cell lines with S-100, HMB-45 and vimentin antibodies was characteristic of melanoma, while staining for epithelial membrane antigen and cytokeratin was negative (data not shown). The CV-1 and 143B TK$^-$ lines used in the propagation of vaccinia virus were also obtained from the ATCC. VMM12-EBV is an Epstein-Barr virus transformed B cell line derived from peripheral blood mononuclear cells (PBMC) of melanoma patient VMM12. Briefly, the PBMC were incubated with filtered supernatant from the EBV producing cell line B-958 for 1 h at 37¡C, followed by culture in RPMI 1640 media with 10% fetal calf serum (FCS) and antibiotics, plus a 1:100 dilution of PHA. K562 is an NK-sensitive human erythroleukemia line. T2-A3 (an HLA-A3 transfectant of the antigen-processing-defective mutant human lymphoid cell line, T2) was provided by P. Cresswell. HLA typing was performed by microcytotoxicity assay on autologous

lymphocytes (Gentrak). Expression of HLA-A3 by tumor cells was confirmed by staining with the monoclonal antibody (MAb) GAP-A3 provided by P. Cresswell.

Production of recombinant vaccinia virus expressing human Pmel-17

The full-length Pmel-17 cDNA was sub-cloned from pcDNA1/neo (Invitrogen) into a modified pSC11 vector adjacent to the vaccinia P7.5 early/late promoter using standard recombinant DNA methods. Standard dideoxy sequencing was used to confirm insertion and orientation. A recombinant vaccinia virus expressing the protein encoded by this gene (vac-Pmel-17) was generated using published methods. Briefly, CV-1 cells were infected with the parental WR strain of vaccinia virus and transfected (Lipofectin, Gibco-BRL) with the pSC11.3-Pmel-17 plasmid. Thymidine-kinase negative recombinants were amplified in 143B TK^- cells in the presence of bromodeoxyuridine (Sigma, St Louis, MO). Recombinants with beta-galactosidase activity were isolated and cloned through several rounds of plaque purification. Large-scale stocks were produced, sucrose purified, and titered in CV-1 cells.

Generation of melanoma-specific cytotoxic T cells: CTL were generated following the detailed protocols previously reported. Malignant melanoma was resected from lymph nodes of patient VMM18. Nodes were mechanically dissociated and enzymatically digested in Eagle's MEM (GIBCO, Grand Island, NY) containing 2.5% FCS, 0.1% collagenase B (Boehringer Mannheim), 0.002% DNAase (Sigma), penicillin 100 U/ml, streptomycin 100 ug/ml (Pen-Strept, GIBCO) at room temperature. T cell lines were established from the mixture of lymphocytes and tumor obtained from the digests, using a ratio of tumor cells to lymphocytes of 1:1. Cells were cultured in 24-well tissue culture plates (Linbro, Hamden, CT) in RPMI 1640 (Sigma) containing 10% FCS, Pen-Strept, and 20 U/ml rIL-2 (Cetus, Emeryville, CA) and were maintained by repeated stimulation with irradiated (10 Gy) fresh cryopreserved autologous tumor cells or with the autologous tumor cell line at a tumor to lymphocyte ratio of 1:10 every 10-12 days. T cell specificity for autologous melanoma was

confirmed after 28 days of culture. Melanoma specific T lymphocytes were then expanded by a modification of methods by E. Goulmy (personal communication), by mixing 1 x 10^6 specific T-cells with 5 x 10^6 irradiated (10 Gy) autologous melanoma stimulators and 10 x 10^6 irradiated (10 Gy) allogenic PBL feeders (pooled from at least three donors). The cells were cultured at 37 °C in 80mls RPMI 1640 containing 10% FCS, Pen-Strept, and 20 U/ml rIL-2 in the edge of an upright T-75 flask (Falcon), set at a 45° angle. After five days 40ml fresh culture medium was added to the flask which was then placed upright for a further two days. T lymphocytes were harvested and cryopreserved in 2 x 10^6 aliquots in 90% FCS/10% DMSO for use in cytotoxic T cell assays. This method was found to permit significant expansion of T-cell numbers without changing the specificity of the CTL line (data not shown). T cells were evaluated by flow cytometry after staining with fluorescein- or phycoerythrein-conjugated antibodies to CD3, CD4, CD8 and CD16 (GenTrak Inc., Plymouth Meeting, PA. and Olympus Corp, Lake Success, NY). Multiple $CD8^+$ VMM18 CTL lines were generated following this protocol with consistent results from each. Similar methods were used for generation of CTL lines from other patients, such as VMM12.

Cytotoxicity assays: Cell mediated lysis of target cells was determined using a standard 4 h ^{51}Cr-release assay. Briefly, ^{51}Cr-labeled target cells were plated at $2x10^3$ cells/well in triplicate on 96-well V-bottom plates (Costar, Cambridge, MA) with indicated ratio of effector cells in a final volume of 200 microliters. Wells containing either culture medium or 1M HCl in place of the effector cells served as spontaneous and maximum ^{51}Cr-release controls, respectively. Plates were centrifuged at 100 x g for 3 min and incubated at 37 °C for 4 h, after which 150 microliters of supernatant from each well was counted on a gamma counter (ICN). The percent specific lysis was calculated using the equation: [(experimental release - spontaneous release) / (maximum release - spontaneous release)] x 100. Vaccinia infected targets were generated by infecting cells with 50 pfu/cell of appropriate recombinant vaccinia virus at 37°C for 5 h, prior to ^{51}Cr-

labeling. Antibody blocking assays were performed by incubating ^{51}Cr-labeled target cells with affinity purified monoclonal antibodies (MAb) for 1 h at 37°C, prior to incubation with effector CTL. The MAbs used included W6/32, specific for a monomorphic determinant on all human class I MHC molecules; L243, specific for a determinant on human DR molecules; and GAP-A3, specific for HLA-A3. For cold target inhibition assays, CTL were incubated with unlabeled (cold) target cells for 1 h at 37°C, prior to addition of ^{51}Cr-labeled (hot) targets.

Reconstitution with synthetic peptides: Peptide sequences were selected from the reported human sequence of Pmel-17/gp100 based on predicted HLA-A3 binding motifs. These peptides were synthesized by standard Fmoc chemistry using a Gilson model AMS422 peptide synthesizer. Peptides were reconstituted in CTL assay medium (RPMI 1640, 10% FCS, antibiotics) and pre-incubated for 2 h with 2×10^3 ^{51}Cr labeled target cells in 100 microliters/well in 96-well plates. Effector cells were added in 100 microliters assay medium for a final effector to target (E:T) ratio of 20:1 and the remainder of the assay was performed as in standard chromium release assays described above. Wells containing peptide and target cells but no CTL were used as controls to rule out toxicity of the peptides themselves. Initial experiments were performed with unpurified synthetic peptides. Biologically active peptides identified at initial screening were then purified to >98% by reversed-phase HPLC on a Vydac C-4 column with 0.05% trifluoroacetic acid:water and an acetonitrile gradient, then re-evaluated in CTL assays.

Isolation of naturally processed HLA-A3 associated peptides. HLA-A3-associated peptides were acid eluted from HLA-A3 molecules affinity-purified from melanoma cells, as previously described for A2-associated peptides. Briefly, VMM18 melanoma cells cultured in cell factories (Nunc, Naperville, IL), were washed three times in cold PBS, pelleted, then snap-frozen. Cell pellets were detergent solubilized in 1% CHAPS, 174 mg/ml PMSF, 5 mg/ml aprotinin, 10 mg/ml leupeptin, 16 mg/ml pepstatin A, 33 mg/ml

iodoacetamide, 0.2% sodium azide and 0.03 mg/ml EDTA for 1 h at 4°C. After centrifugation at 100,000 x g for 1 h at 4°C, the pellet of insoluble proteins was discarded, and the supernatant was filtered (0.2 um), then passed over a protein A-Sepharose column precoated with MAb GAP-A3. HLA-A3 molecules and associated peptides, bound to GAP-A3, were then eluted with 0.2 N acetic acid, pH 2.7, then peptides were dissociated at pH 2.1 by bringing the solution to 10% acetic acid followed by boiling for 5 min. Finally, peptides were centrifuged through Ultrafree-CL 5000 -KDa filters (Millipore, Bedford, MA) at 2500 x g for 5 h. Filtrates containing purified peptides were concentrated using vacuum centrifugation and stored at -80°C.

HPLC fractionation and co-elution of naturally processed and synthetic peptides: Extracted HLA-A3 associated peptides were fractionated by reversed-phase HPLC on a Brownlee narrowbore C-18 Aquapore column (2.1 mm x 3cm, A, 7mm) and eluted with a 40-minute gradient of 0 to 60% (v/v) acetonitrile/0.085% TFA in 0.1% TFA. Fractions were collected at 1 minute intervals. A synthetic peptide, ALLAVGATK, was eluted under identical conditions to identify its elution point.

Peptide identification and sequencing by mass spectrometry: Isolated peptides were loaded onto a C18 microcapillary column (75m i.d. x 12 cm) and gradient-eluted using acetonitrile and 0.1M acetic acid, with the concentration of acetonitrile increasing at 2%/min, into a Finnigan-MAT TSQ-7000 (San Jose, California) triple quadrupole mass spectrometer equipped with an electrospray ion source. For mass spectrometric peptide sequencing, collision activated dissociation (CAD) mass spectra were recorded for *m/z* 423.

<u>*Results*</u>

HLA-A3 restricted melanoma specific human CTL recognize one or more commonly expressed antigens

Cytotoxic T lymphocyte (CTL) lines were generated by repeated co-culture of lymphocytes, originally harvested from a tumor involved lymph node, with fresh or cultured

autologous melanoma cells from patient VMM18 in the presence of rIL-2 as described. Several $CD3^+$, $CD8^+$, $CD4^-$ CTL lines were derived, which lysed autologous tumor, whereas there was minimal lysis of the NK target K562, an allogeneic HLA-$A3^+$ EBV-transformed B cell line (VMM12-EBV) or the HLA-$A3^-$ melanoma DM6 (Fig.1A). Lysis of autologous tumor was MHC-class I restricted, based on inhibition with W6/32, a MAb specific for human class I molecules, but not L243, a MAb specific for a determinant on human DR molecules (Fig.1B). Furthermore, inhibition observed with GAP-A3, a MAb recognizing HLA-A3, demonstrates that the VMM18 CTL recognize one or more peptides presented by HLA-A3 on the surface of the autologous melanoma cells.

VMM18 CTL lysed several other HLA-A3 matched allogeneic melanomas: VMM1, VMM12, DM122, and SkMel-2, indicating that one or more shared epitope(s) are presented on the surface of multiple HLA-$A3^+$ melanomas (Table 101). In cold target inhibition assays, lysis of allogeneic HLA-A3 matched melanoma cells by VMM18 CTL was inhibited by unlabeled (cold) autologous melanoma cells (VMM18), but not by HLA-$A3^-$ melanoma cells (DM6) (Fig. 2). This confirms the existence of shared epitopes restricted by HLA-A3. Lysis of HLA-$A3^+$ non-melanoma cells such as the squamous lung cancer cell line SkMes-1 and the lymphoblastoid cell line VMM12-EBV was not observed (Table 101), indicating that these epitopes may be derived from one or more melanoma-specific proteins.

Identification of an HLA-A3 restricted peptide from the melanocyte differentiation antigen Pmel-17/gp100

It has been observed that expression by melanoma cells of the melanocyte differentiation antigen Pmel-17 correlates with recognition by HLA-A2 restricted melanoma specific CTL. All of the HLA-$A3^+$ melanoma lines recognized by VMM18 CTL express Pmel-17, as determined by immunohistochemical staining with antibodies HMB-45 and NKI-beteb. Significantly, VMM34 melanoma cells which are also HLA-$A3^+$ but negative for Pmel-17 expression, were not recognized by VMM18 CTL.

To determine whether Pmel-17 encodes an epitope

recognized by HLA-A3 restricted CTL, a recombinant vaccinia virus (vac-Pmel-17) expressing the full-length protein encoded by the Pmel-17 cDNA was constructed. Expression of Pmel-17 by the recombinant vaccinia was confirmed by infecting C1R-A2, an HLA-A2$^+$ non-melanoma cell line, with vac-Pmel-17 or an irrelevant recombinant vaccinia encoding the influenza nucleoprotein, NP (vac-NP). Only the vac-Pmel-17 infected cells were lysed by VMM5 CTL, previously demonstrated to recognize an HLA-A2 restricted peptide derived from this antigen (data not shown). When HLA-A3$^+$ VMM12-EBV cells were infected with vac-Pmel-17, they were lysed by VMM18 CTL. Whereas uninfected VMM12-EBV cells, and cells infected with a control recombinant vaccinia virus (vac-NP), were not recognized (Fig.3). Therefore, expression of Pmel-17/gp100 by VMM12-EBV cells made these cells targets for lysis by VMM18 CTL, suggesting that the CTL recognized a peptide derived from Pmel-17/gp100 and presented by HLA-A3.

Thirty-four peptides from Pmel-17/gp100 were synthesized on the basis of peptide binding motifs for HLA-A3. These peptides were screened for their ability to sensitize allogeneic HLA-A3$^+$ non-melanoma cells for lysis by VMM18 CTL. Two of these peptides, the nonamer ALLAVGATK and its amino terminal truncated octamer LLAVGATK, sensitized VMM12-EBV for lysis by VMM18 CTL (Table 102). The relative ability of these peptides to sensitize targets for lysis was determined in a titration assay using T2-A3, the non-melanoma HLA-A3 transfectant of the antigen processing defective mutant cell line T2. Half maximal lysis was induced with 1-10 nM and > 1 uM of peptides ALLAVGATK and LLAVGATK respectively, while recognition of the HLA-A3 binding peptide QVPLRPMTYK, derived from the HIV Nef protein was not observed (Fig. 4). The nonamer peptide was able to sensitize targets for VMM18 CTL recognition at a significantly lower concentration than the octamer, suggesting that it is more likely to be the naturally processed peptide to which the CTL were primed.

The nonamer peptide ALLAVGATK is naturally processed and presented by melanoma cells in association with HLA-A3

To confirm that the HLA-A3 restricted peptide ALLAVGATK from Pmel-17/gp100 was naturally processed, HLA-A3 associated peptides were isolated from VMM18 melanoma cells and fractionated by reversed-phase HPLC, as described. The synthetic peptide ALLAVGATK (mass of 846 and *m/z* of 423) was eluted under identical conditions and found in fraction 14. Collision activated dissociation (CAD) sequencing of the peptide(s) *m/z* 423 was subsequently performed on the HLA-A3 associated peptides eluted in fraction number 14 from VMM18 melanoma cells, confirming its amino acid sequence as ALLAVGATK, identical to the predicted synthetic peptide. This confirms that peptide ALLAVGATK from Pmel-17/gp100 is a naturally processed antigenic peptide, presented by HLA-A3 on melanoma cells.

Discussion

Evidence of HLA-A3 restricted recognition of melanoma cells by melanoma specific CTL has been previously observed however, melanoma antigens presented by HLA-A3 were not previously identified. In the present report, we have corroborated the previous finding by demonstrating the existence of shared melanoma antigens restricted by HLA-A3. We have also identified a specific naturally-processed peptide, ALLAVGATK, derived from Pmel-17, as an epitope recognized by HLA-A3 restricted melanoma specific CTL from patient VMM18. Since this protein, Pmel-17, is expressed by the majority of melanoma cells and is a tissue differentiation antigen of melanocytic origin, this peptide represents a shared epitope for A3-restricted melanoma-specific CTL.

Analysis of HLA-A2 associated peptides eluted from the surface of melanoma cells has demonstrated that the amino acid sequences of naturally processed MHC-associated peptides may differ from their respective gene-encoded amino acid sequences because of post-translational modifications and that the gene-encoded sequence may not be presented at all. To confirm that the predicted peptide, ALLAVGATK, is naturally processed, HLA-A3 associated peptides from VMM18

tumor cells were evaluated directly and sequenced by tandem mass spectrometry. By this method, it has been confirmed that this peptide is naturally processed and presented by HLA-A3.

HLA-A2 and -A3 are two of the most commonly expressed haplotypes in Caucasian populations, representing 46% and 24% respectively. The identification of an HLA-A3 restricted epitope expands the number of patients (to 60%) who might be targeted for immunization against Pmel-17 antigens. It also suggests that Pmel-17 directed immunotherapy may be an important part of immune therapy for melanoma patients of many different haplotypes.

Although the Pmel-17 derived peptide ALLAVGATK is recognized by VMM18 CTL, it is not recognized by CTL from another patient, VMM12. However, VMM12 CTL do recognize and lyse VMM18 melanoma cells. Because the only Class I MHC molecule shared by VMM12 and VMM18 is HLA-A3, it is evident that at least one additional shared CTL epitope is expressed by both of these tumors.

Expt No.				#1	#2	#3
Target cell	Cell Type	HLA-A3 expression	Pmel-17 expression	% Specific Lysis		
VMM18	Melanoma	+	+	94	88	55
VMM12	Melanoma	+	+	79	66	62
VMM1	Melanoma	+	+	57	57	40
DM122	Melanoma	+	+	78	51	39
SkMel-2	Melanoma	+	+	52	51	ND
VMM12-EBV	Lympho-blastoid	+	-	3	5	ND
K562	NK target	-	-	8	ND	7
SkMes-1	Lung CA	+	-	ND	7	ND
DM6	Melanoma	-	+	ND	6	6

Table 101. Recognition of autologous and allogeneic HLA-A3+ melanoma cell lines by VMM18 CTL. Targets were assayed in triplicate using an E:T ratio of 10:1 in the three representative experiments shown. The known HLA haplotypes of the melanoma lines are as follows: VMM18 (A3, 31, B60, C3); VMM12 (A1, A3, B7, B14); VMM1 (A3, A26, B51); DM122 (A3, A33, B7, B18); SkMel-2 (A3, determined by FACS analysis using MAb GAP-A3); DM6 (A2.1, B12, B13, C1, C2). ND=not determined in this experiment.

synthetic peptide	% specific lysis	
	VMM18 CTL	peptide alone
QLRALDGGNK	4	7
ALQLHDPSGY	10	8
AVPSGEGDAF	7	5
TVSCQGGLPK	10	5
QILKGGSGTY	0	5
SLIYRRRLMK	5	10
PLAHSSSAF	2	8
ALDGGNKHF	4	3
FLRNOPLTF	-4	-2
YLAEADLSY	-2	8
QVPLDCVLY	5	6
PLDCVLYRY	1	2
CVLYRYGSF	-1	9
QLVLHQILK	-3	-4
ILKGGSGTY	-5	-3
AVVLASLIY	2	-3
LIYRRRLMK	2	-2
ALLAVGATK	27	1
GVSRQLRTK	0	-8
TLIGANASF	0	-4
ALNFPGSQK	-1	8
QVWGGQPVY	-6	-2
YVWKTWGQY	-2	2
ASFSIALNF	2	1
LLAVGATK	14	3
ALVVTHTY	6	-1
LNFPGSQK	-4	-1
TITDQVPF	-3	-3
QLHDPSGY	-2	-3
DLSYTWDF	-5	0
VLYRYGSF	-1	-3
LVLHQILK	-6	-3
VVLASLIY	2	-1
WLRLPRIF	-2	-5

Table 102. Recognition of synthetic Pmel-17 peptides. Thirty-four peptides (ten octamers, eighteen nonamers, and six decamers) were synthesized and tested for their ability to reconstitute specific lysis of the HLA-A3+ non-melanoma cell line VMM12-EBV in a 4 h ^{51}Cr release assay. Labeled targets were incubated with each peptide at approximately 10ng/ml for 2 h at 37^{0}C prior to the addition of VMM18 CTL at a final E:T of 20:1. Specific lysis of autologous tumor was 51%, lysis of VMM12-EBV alone was 5%. Similar results were obtained with a 100-fold higher concentration of peptide.

References for Example IX

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EXAMPLE X

A recombinant vaccinia virus has been constructed that was designed to express the full-length tyrosinase protein. Appropriate expression of the tyrosinase protein was confirmed by infecting tyrosinase-negative non-melanoma cells with this newly constructed virus and demonstrating their subsequent recognition by murine tyrosinase specific T cells. Human HLA-A2-positive lymphoblastoid cells (JY) were infected with a recombinant vaccinia virus expressing the full-length tyrosinase protein, labeled, and combined with murine cytolytic T cells specific for the HLA-A2-restricted tyrosinase "D" peptide (YMDGTMSQV), which were generated in our laboratories. Recognition of the vaccinia encoded tyrosinase was ascertained by measuring target cell lysis in a standard chromium release assay. As expected, uninfected JY, and JY infected with a recombinant vaccinia encoding an irrelevant protein (NP), were not recognized. JY cells pulsed with the "D" peptide and DM6 melanoma cells served as positive controls, demonstrating the lytic potential and specificity of the T cells in this particular assay, as well as the efficacy of the vaccinia construct as a means of inducing expression of tyrosinase in a cell.

A panel of human cytolytic T lymphocytes (CTL) was then screened for recognition of the tyrosinase protein by the same method. One human CTL line, VMM12, was found to specifically recognize tyrosinase. This experiment was performed as above, except that the CTL were derived from patient VMM12, and the tyrosinase-negative non-melanoma human B lymphoblastoid cell line VMM12EBV served as the target cell. Recognition of VMM12 melanoma tumor cells verifies the

lytic potential of these CTL. VMM12EBV infected with recombinant vaccinia encoding tyrosinase were recognized and lysed, whereas VMM12EBV infected with a recombinant vaccinia construct encoding an irrelevant protein (NP) were not recognized, demonstrating that the recognition of VMM12EBV infected with vaccinia-tyrosinase was absolutely dependent on expression of the tyrosinase protein.

The specific Major Histocompatability Complex (MHC) molecule recognized by VMM12 CTL in association with the tyrosinase epitope was determined by repeating the previously described experiment using target cells (C1R) expressing individual MHC molecules. Only those targets which shared expression of HLA-A1 with VMM12 were recognized, demonstrating HLA-A1 as the "restriction element". This experiment was also performed as above, except that additional target cells, expressing individual HLA molecules shared with VMM12EBV (A1, A3 & B7), were included. As observed with VMM12EBV infected with irrelevant vaccinia viruses (above), uninfected VMM12EBV and uninfected C1R (non-melanoma) cells were not recognized, as expected. VMM12EBV (which express the HLA-A1,-A3,-B7, and -B14 MHC molecules) infected with the tyrosinase-expressing recombinant vaccinia virus, and VMM12 melanoma tumor cells, were recognized. The only C1R (lymphoid) target cells that were recognized were those that expressed both HLA-A1 and tyrosinase.

Example XI

Identification of a Tyrosinase Epitope Recognized by Human Melanoma-Reactive, HLA-A1 Restricted CTLs.

Introduction

We have identified the peptide KCDICTDEY (K is N-terminal), from the tyrosinase protein, as an epitope for HLA-A1-restricted melanoma-specific cytotoxic T-lymphocytes (CTL). This work has been done by generating HLA-A1-restricted melanoma-reactive CTL, creating a vaccinia construct encoding the intact human tyrosinase gene, then infecting HLA-A1+ non-melanoma target cells with the vac-tyrosinase construct. In doing so, VMM12 CTL and VMM15 CTL both recognize an HLA-A1-associated peptide derived from

tyrosinase. We have since screened a large panel of peptides that we predicted to bind to HLA-A1, from the defined sequence of tyrosinase. The peptide KCDICTDEY, when pulsed onto HLA-A1+ non-melanoma cells (C1R-A1), reconstitutes an epitope for VMM15 CTL. To a lesser extent, two other peptides that are longer than 9-residues, but which contain the entire KCDICTDEY sequence, also reconstitute an epitope for these CTL. None of 116 other peptides tested worked. Thus, we believe this is an epitope which can be used as an immunogen in treating or preventing melanoma in the 20-25% of patients who express HLA-A1.

Cell lines and HLA typing: The human melanoma cell lines VMM1, VMM12, VMM15, VMM18, VMM30 and VMM34 were derived from patients at the University of Virginia (Charlottesville, VA). Other fresh (uncultured) tumors VMM14 and VMM21 were also prepared from surgical specimens from patients at the University of Virginia. DM6 was provided by Drs. H.F. Seigler and T.L. Darrow at Duke University (Durham, NC). Immunohistochemical staining of these cell lines with S-100, HMB-45 and vimentin antibodies was characteristic of melanoma, while staining for epithelial membrane antigen and cytokeratin was negative. The CV-1 and 143B TK^- lines used in the propagation of vaccinia virus were also obtained from the American Type Culture Collection (ATCC, Bethesda, MD). VMM12-EBV is an Epstein-Barr virus transformed B cell line derived from peripheral blood mononuclear cells (PBMC) of melanoma patient VMM12. Briefly, the PBMC were incubated with filtered supernatant from the EBV producing cell line B-958 for 1 h at 37°C, followed by culture in RPMI 1640 media with 10% fetal calf serum (FCS) and antibiotics, plus a 1:100 dilution of PHA. K562 is an NK-sensitive human erythroleukemia line. T2-A3 (an HLA-A3 transfectant of the antigen-processing-defective mutant human lymphoid cell line, T2) was provided by P. Cresswell. HLA typing was performed by microcytotoxicity assay on autologous lymphocytes (Gentrak). Expression of HLA-A1 by tumor cells was confirmed by staining with a monoclonal antibody (MAb) from One Lambda.

CTL lines: We have generated human melanoma-specific CTL

lines by in vitro stimulation with autologous tumor, from patients whose tumors express melanocytic tissue differentiation antigens and express one or more of the MHC molecules A1, A3, B7, and B8. Methods for CTL generation have been described. (Table 111 and Figure 5).

Production of recombinant vaccinia virus expressing the human genes encoding melanocytic tissue differentiation antigens: We have examined class I MHC-associated epitopes for the melanocytic tissue differentiation antigens by using vaccinia constructs for each of the genes Pmel17/gp100, tyrosinase, and MART-1/MelanA. A cDNA clone of the Pmel17 gene (HUMPMEL17 - Genbank) was generously provided by S.N. Wagner, Essen, Germany. The tyrosinase gene was provided by Thierry Boon, Brussels. We have PCR cloned out a cDNA clone of the MART-1/Melan-A gene from DM6 melanoma cells. The entire open-reading frame for each of these cDNA's was sub-cloned into a modified pSC11 vector (Ref Hahn JEM 1991) adjacent to the vaccinia P7.5 early/late promoter using standard recombinant DNA methods. Standard dideoxy sequencing was used to confirm insertion and orientation. A recombinant vaccinia virus expressing the protein encoded by this gene (vac-Pmel-17) was generated using published methods (Ref Macket J.Virol 1984). Briefly, CV-1 cells were infected with the parental WR strain of vaccinia virus and transfected (Lipofectin, Gibco-BRL) with the pSC11.3-Pmel-17 plasmid. Thymidine-kinase negative recombinants were amplified in 143B TK^- cells in the presence of bromodeoxyuridine (Sigma). Recombinants with beta-galactosidase activity were isolated and cloned through several rounds of plaque purification. Large-scale stocks were produced, sucrose purified, and titered in CV-1 cells.

The resulting recombinant vaccinia viruses were used to infect the lymphoblastoid cell lines C1R-A1, C1R-A2, C1R-A3, C1R-B7, and C1R-B8, where C1R is a human lymphoblastoid line devoid of native expression of HLA-A or HLA-B region molecules, but expressing low levels of HLA-C and MHC Class II molecules. In some cases EBV-transformed B cells with defined MHC expression were used for the infections. This resulted in transient expression of the antigens of interest.

These cells were assayed for recognition by CTL in Cr51-release assays. As a negative control, target cells were also infected with a recombinant vaccinia virus with an irrelevant DNA insert (influenza nucleoprotein, NP). Thus, the cell lines listed above permit isolated evaluation of the expression of antigenic peptides in association with the common Class I MHC molecules HLA-A1, A2, A3, B7, and B8.

Evaluating recognition of target cells by CTL.

Reactivity was assessed by cytotoxicity in a 4-hour chromium release assay. Positive controls were the autologous tumor and known cross-reactive tumor lines. A negative control was uninfected C1R-MHC line and a C1R-MHC line transfected with a vaccinia construct expressing influenza nucleoprotein, vac-NP only. Briefly, ^{51}Cr-labeled target cells were plated at 1 - $2x10^3$ cells/well in triplicate on 96-well V-bottom plates (Costar, Cambridge, MA) with indicated ratio of effector cells in a final volume of 200 microliters. Wells containing either culture medium or 1M HCl in place of the effector cells served as spontaneous and maximum ^{51}Cr-release controls, respectively. Plates were centrifuged at 100 x g for 3 min and incubated at 37 °C for 4 h, after which 150 microliters of supernatant from each well was counted on a gamma counter (ICN). The percent specific lysis was calculated using the equation: [(experimental release - spontaneous release) / (maximum release - spontaneous release)] x 100. Vaccinia infected targets were generated by infecting cells with 50 pfu/cell of appropriate recombinant vaccinia virus at 37°C for 5 h, prior to ^{51}Cr-labeling.

Peptide synthesis and Reconstitution with synthetic peptides:

Peptide sequences were selected from the reported human sequence of tyrosinase, based on predicted HLA-A1 binding motifs (see table 10). These peptides were synthesized by standard Fmoc chemistry using a Gilson model AMS422 peptide synthesizer. Peptides were reconstituted in CTL assay medium (RPMI 1640, 10% FCS, antibiotics) and pre-incubated for 2 h with $2x10^3$ ^{51}Cr labeled target cells in 100 microliters/well in 96-well plates. Effector cells were added in 100 microliters assay medium for a final effector to target (E:T) ratio of

20:1 and the remainder of the assay was performed as in standard chromium release assays described above. Wells containing peptide and target cells but no CTL were used as controls to rule out toxicity of the peptides themselves. Initial experiments were performed with unpurified synthetic peptides. Biologically active peptides identified at initial screening were then purified to >98% by reversed-phase HPLC on a Vydac C-4 column with 0.05% trifluoroacetic acid:water and an acetonitrile gradient, then re-evaluated in CTL assays.

Results

Melanoma-reactive CTL lines recognize MHC-associated peptides from several melanocytic differentiation antigens

The CTL lines listed in Table 111 were evaluated for recognition of peptides derived from the 3 melanocytic tissue differentiation antigens listed above, in chromium-release assays, by transient infection with vaccinia constructs encoding those genes. Examples of their reactivity against HLA-matched allogeneic melanomas are shown in Figure 5. A summary of these results with vaccinia constructs are listed in Table 112 and are shown in Figure 6. Responses to tyrosinase peptides were observed in half of cases. In addition, responses to MART-1 and gp100 peptides were observed in a smaller set of CTL lines.

At least two of the HLA-A1+ CTL lines recognized tyrosinase peptides in an HLA-A1-restricted manner.

VMM12 CTL and VMM15 CTL were assayed initially on autologous EBV-B cells as targets. Reactivity against tyrosinase was observed, so additional studies were performed to confirm the reactivity and to determine the MHC restriction (Figure 6). C1R cells that express selected Class I MHC molecules only were used as target cells. As seen in Figure 6, C1R-A1 cells infected with vac-tyrosinase are recognized by VMM12 and VMM15 CTL, confirming that one or more tyrosinase-derived peptides are recognized by VMM12 and VMM15 CTL in association with HLA-A1.

The peptide representing residues 243-251 of tyrosinase reconstitutes an epitope for VMM15 CTL.

A set of peptides were synthesized from the defined amino acid sequence of tyrosinase, including 9-mers and longer peptides, with tyrosine (Y) at the C-terminal position and T, S, or M at position 2 and/or D, E, A, or S at position 3 (Figure 7). These were assayed for their ability to reconstitute epitopes for melanoma-reactive CTL VMM12 and VMM15. C1R-A1 cells were pulsed with the peptide at concentrations ranging from 0.1 to 10 uM in normal assay medium (RPMI + 10% FCS), then evaluated for recognition in a chromium-release assay. As shown in Figure 8, three peptides were recognized by VMM15 CTL, all containing the sequence KCDICTDEY (tyrosinase residues 243-251). The most effective, even at the lowest concentration tested, was the 9-mer peptide KCDICTDEY, but also recognized were a ten-mer, EKCDICTDEY, and a 13-mer, DAEKCDICTDEY (Figure 8).

Similar reactivity was seen with VMM12 CTL as well, suggesting that KCDICTDEY is a shared antigen on human melanoma cells expressing HLA-A1, against which multiple patients' CTL may be expected to react (Figure 9). The location of this peptide in the intact protein tyrosinase is shown in Figure 10.

Discussion

The peptide KCDICTDEY appears to be recognized by CTL from at least two different patients, in association with HLA-A1. Although longer peptides also are reactive, the dominant response seems to be to KCDICTDEY. This peptide is unusual in its large number of polar amino acid residues, including two aspartic acid residues, one glutamic acid residue, and two cystine residues. The tyrosine residue at position 9 and the aspartic acid at position 3 are important for binding to the MHC. By a computerized system for predicting the binding affinity of individual peptides to HLA-A1 (and other HLA haplotypes), see http://bimas.dcrt.nih.gov:80/cgi-bin/molbio/ken_parker_comboform (The algorithm for this software is discussed in Parker, et al., J. Immunol., 152:163 (1994)), this peptide is predicted to the be the tyrosinase

peptide with highest affinity for HLA-A1, which may make it useful for immunization after pulsing on antigen-presenting cells.

One concern with this peptide is the presence of two cystine residues, which may be susceptible to interaction with other sulfhydryl groups on biologic molecules in vitro and in vivo. Studies on the possibility of this interaction and its effect on CTL recognition are underway. KCDICTDEY is associated with half-maximal lysis at approximately 1 ug/ml (1 uM). Evaluating the possibility of increasing the potency of this activity is underway, by assessing various amino acid substitutions and their effects on CTL recognition.

There have been two peptides described as epitopes for melanoma-reactive HLA-A1-restricted CTL. They are the MAGE-1 and MAGE-3 peptides EADPTGHSY and EVDPIGHLY While these have substantial potential value as immunogens, only a subset of melanoma patients express them. Most other MHC-associated peptide epitopes are HLA-A2 associated. However, HLA-A1 is expressed in approximately 29% of patients in this country. We have previously described an HLA-A3-associated epitope from gp100, ALLAVGATK. Now, with defined peptide epitopes known, it is possible to consider the use of a multivalent peptide vaccine, where all patients expressing either HLA-A1, HLA-A2, or HLA-A3, which is approximately 70% of the patients at risk, may be treated with specific vaccine therapy.

Table 111. CTL lines studied for recognition of target cells infected with vaccinia constructs encoding Pmel17/gp100, Tyrosinase, or MART-1/MelanA

Melanoma Patient ID	Class I MHC expression	Target cell	MHC shared with target	Pmel17-reactive	Tyrosinase-reactive	MART1-reactive
VMM12	A1, A3, B7, B14	VMM12-EBV	A1, A3, B7, B14	0	Yes	---
		VMM15-EBV	A1	0	Yes	0
		C1R-A1	A1	---	Yes	---
		C1R-A3	A3	---	0	---
		C1R-B7	B7	---	0	---
VMM15	A1, A25, B8, B18	VMM15-EBV	A1, A25, B8, B18	0	Yes	Yes
		C1R-A1	A1	---	Yes	---
		C1R-B8	B8	---	+/-	---
		VMM38-EBV	B18	---	0	---
VMM10	A3, A25, B62, C1, C4	VMM15-EBV	A25	0	+/-	+/-
		VMM12-EBV	A3	0	0	0
		VMM16-EBV	C1, C4	0	0	0

VMM30	A1, A2, B27, B57, C2, C6	VMM30-EBV	A1, A2, B27, B57, C3	0	Yes	0
VMM14	A1, A25, B8, B48	VMM15-EBV	A1, A25, B8	0	Yes	0
VMM21	A1, A2, B7, B37	VMM21-EBV	A1, A2, B7, B37	0	0	Yes
VMM18	A3, A31/33, B60, C3	VMM18-EBV	A3, A31/33, B60, C3	Yes	0	Yes
		VMM12-EBV	A3	Yes	0	---
		C1R-A3	A3	Yes	---	Yes
		VMM17-EBV	A33?	0	---	0
VMM19	A24, B35, B55	VMM19-EBV	A24, B35, B55	+/-	0	0
DM331	A1, A2, B15, B62	VMM12-EBV	A1, A2, B15, B62	0	0	---
VMM39	A2, A3, B7, B44	VMM12-EBV	A3, B7	0	0	0
		VMM30-EBV	A2	0	0	+/-

--- = not tested

+/- = results are equivocal and need further investigation.

Table 112. Summary of CTL reactivities observed

Patient ID	Source of CTL epitope	Restricting Class I MHC molecule
VMM10	------------	n/a
VMM12	Tyrosinase	A1
VMM14	Tyrosinase	unknown
VMM15	Tyrosinase	A1
	MART-1	unknown
VMM18	Pmel17	A3
	MART-1	A3
VMM19	------------	n/a
VMM21	MART-1	unknown
VMM30	Tyrosinase	unknown
VMM39	------------	n/a
DM 331	------------	n/a

REMARKS

All references cited herein, including journal articles or abstracts, published or corresponding U.S. or foreign patent applications, issued U.S. or foreign patents, or any other references, are entirely incorporated by reference herein, including all data, tables, figures, and text presented in the cited references. Additionally, the contents of the references cited within the references cited herein are also entirely incorporated by reference.

Reference to known method steps, conventional methods steps, known methods or conventional methods is not in any way an admission that any aspect, description or embodiment of the present invention is disclosed, taught or suggested in the relevant art.

The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying knowledge within the skill of the art (including the contents of the references cited herein), readily modify and/or adapt for various applications such specific embodiments, without undue experimentation, without departing from the generic concept of the present invention. Therefore, such adaptations and modifications are intended to be comprehended within the meaning and range of equivalents of the disclosed embodiments, based on the teaching and guidance presented herein. It is to be understood that the phraseology or terminology herein is for the purpose of description and not of limitation, such that the terminology or phraseology of the present specification is to be interpreted by the skilled artisan in light of the teachings and guidance presented herein.

For immunological techniques generally, see Coligan, et al, Current Protocols in Immunology (NIH: 994); Harlow and Lane, Antibodies:A laboratory Manual (Cold Spring Harbor Lab.: 1988).

An immunogen is deemed not to occur in nature, even though its component epitopes do occur in nature, if the immunogen itself, as a single molecule, does not occur in nature. For example, a conjugate of 946L to albumin does not occur in nature even though 946L is a fragment of pMel-17

which is generated by the immune system processing of pMel-17 and complexes with MHC.

What is claimed is:

1. A melanoma-specific immunogen which comprises one or more melanoma-specific CTL epitopes which may be the same or different, wherein at least one of said epitopes is at least substantially homologous to a CTL epitope selected from the group consisting of HLA-A1- and HLA-A3-restricted epitopes of a melanoma antigen selected from the group consisting of pMel-17 and tyrosinase.

2. The immunogen of claim 1 which further comprises an epitope which is at least substantially homologous with an HLA-A2-restricted CTL epitope of a melanoma antigen.

3. The immunogen of claim 2 in which at least one HLA-A2 epitope is a pMel-17 epitope.

4. The immunogen of claim 3 in which said HLA-A2, pMel-17 epitope is at least substantialy homologous with peptide 946L (SEQ ID NO:14).

5. The immunogen of claim 4 in which said HLA-A2 epitope is peptide 946I or peptide 946L.

6. The immunogen of any of claims 2-5 in which at least one HLA-A2 epitope is a tyrosinase epitope.

7. The immunogen of claim 6 in which the HLA-A2 epitope is peptide 1030 (SEQ ID NO:9).

8. The immunogen of any of claims 1-6 wherein at least one of said CTL epitopes is an HLA-A1 epitope.

9. The immunogen of claim 8 wherein at least one HLA-A1 epitope is a tyrosinase epitope.

10. The immunogen of claim 9 in which at least one HLA-A1 epitope is at least substantially homologous with KCDICTDEY.

11. The immunogen of claim 10 wherein at least one HLA-A1 epitope is identical to KCDICTDEY.

12. The immunogen of any of claims 1-11 wherein at least one of said CTL epitopes is an HLA-A3 epitope.

13. The immunogen of claim 12 in which at least one HlA-A3 epitope is a pMel-17 epitope.

14. The immunogen of claim 13 in which at least one HLA-A3 epitope is at least substantially homologous with ALLAVGATK.

15. The immunogen of claim 14 in which at least one HLA-A3 epitope is identical to ALLAVGATK.

16. The immunogen of any of claims 1-15 which comprises both at least one HLA-A1-restricted epitope and at least one HLA-A3-restricted epitope.

17. The immunogen of claim 16 which further comprises at least one HLA-A2-restricted epitope.

18. The immunogen of any of claims 1-17, which is an isolated, purified, or synthetic peptide of about 9 to about 15 residues in length.

19. An expression vector which comprises a gene encoding a melanoma-specific immunogen according to any of claims 1-18, operably linked to one or more expression control sequences, whereby said gene may be expressed in a mammalian subject, in which subject said expressed immunogen is capable of eliciting a melanoma-specific CTL response.

20. A composition comprising an immunogen according to any of claims 1-18 and a class I MHC molecule, whereby T lymphocytes may be stimulated by said peptide.

21. T lymphocytes stimulated by an immunogen according to any of claims 1-18.

22. A method of protecting a mammal against melanoma which comprises administering to a mammal in need thereof a therapeutically or prophylactically effective amount of one or more of:

(a) The immunogen of any of claims 1-18,

(b) an expression vector which comprises a gene encoding a melanoma-specific immunogen according to any of claims 1-18, operably linked to one or more expression control sequences, whereby said gene may be expressed in a mammalian subject, in which subject said expressed immunogen is capable of eliciting a melanoma-specific CTL response, or

(c) T lymphocytes stimulated by an immunogen according to any of claims 1-18.

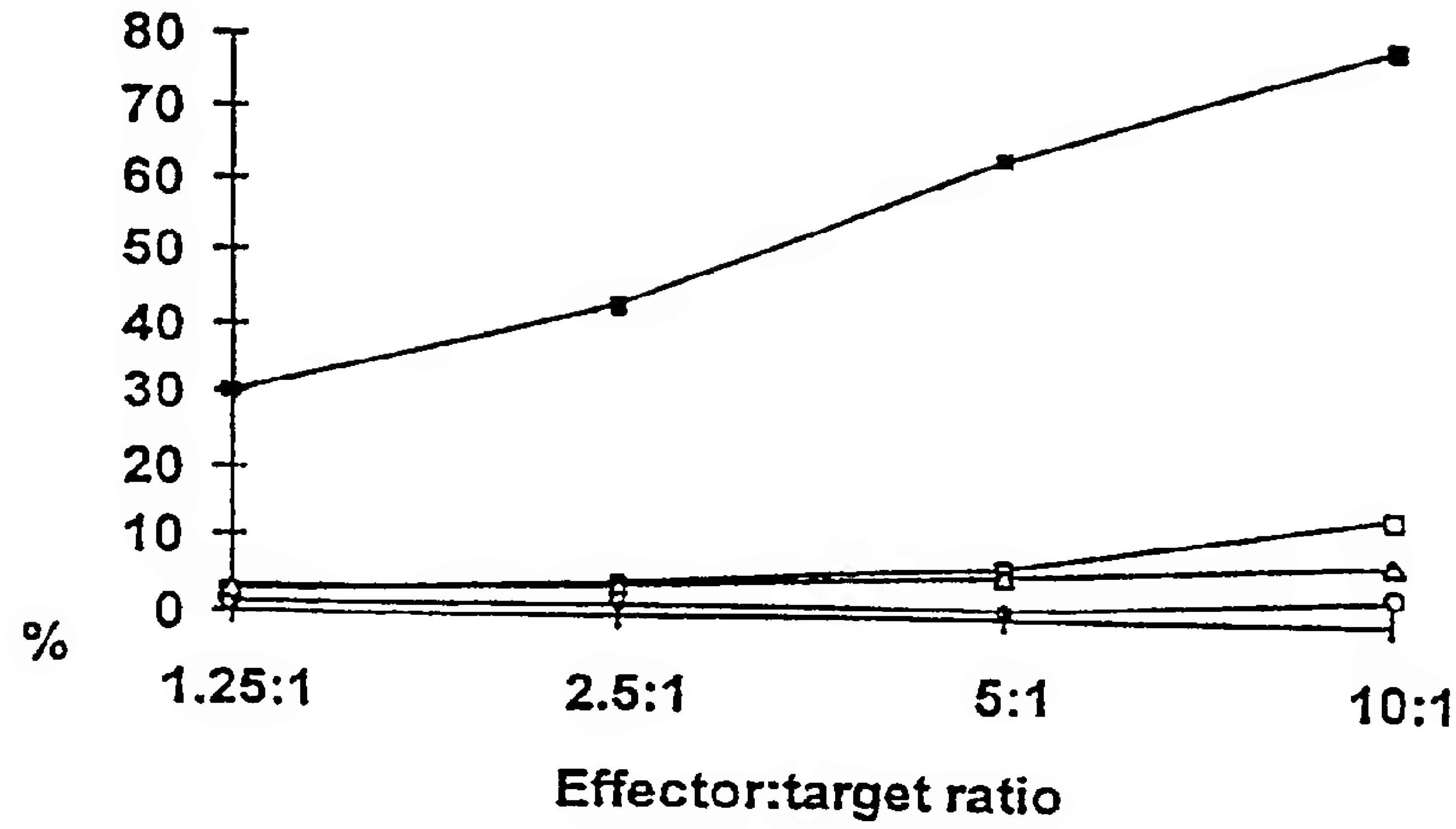


Figure 1A Melanoma specific recognition of autologous tumor by VMM18 CTL. VMM18 cells (solid squares) were lysed by the CTL in a 4 h ^{51}Cr release assay, while minimal lysis of non-melanoma targets K562 (open squares), VMM12-EBV (open circles) and the HLA-A3^{-} melanoma DM6 (open triangles) was observed.

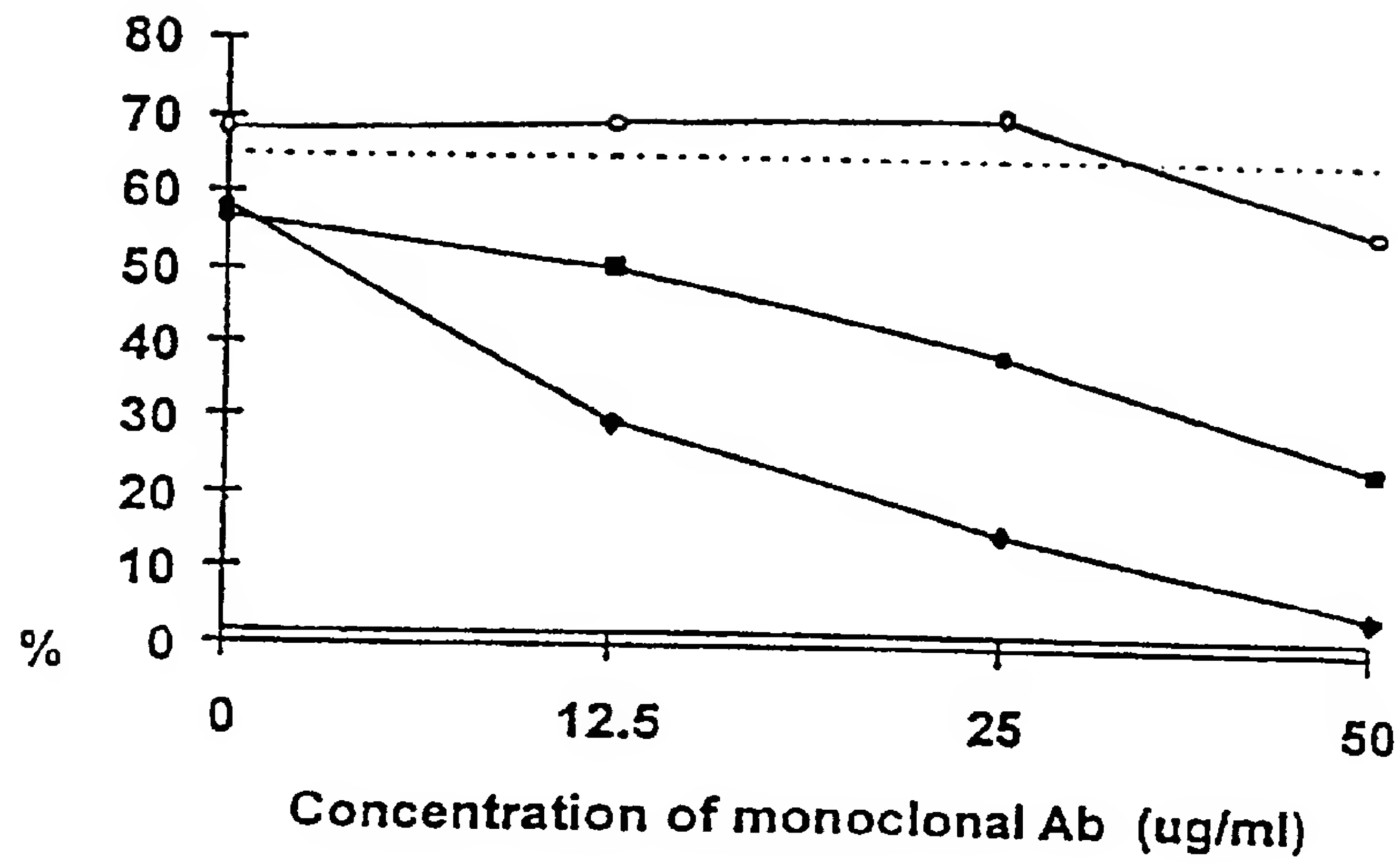
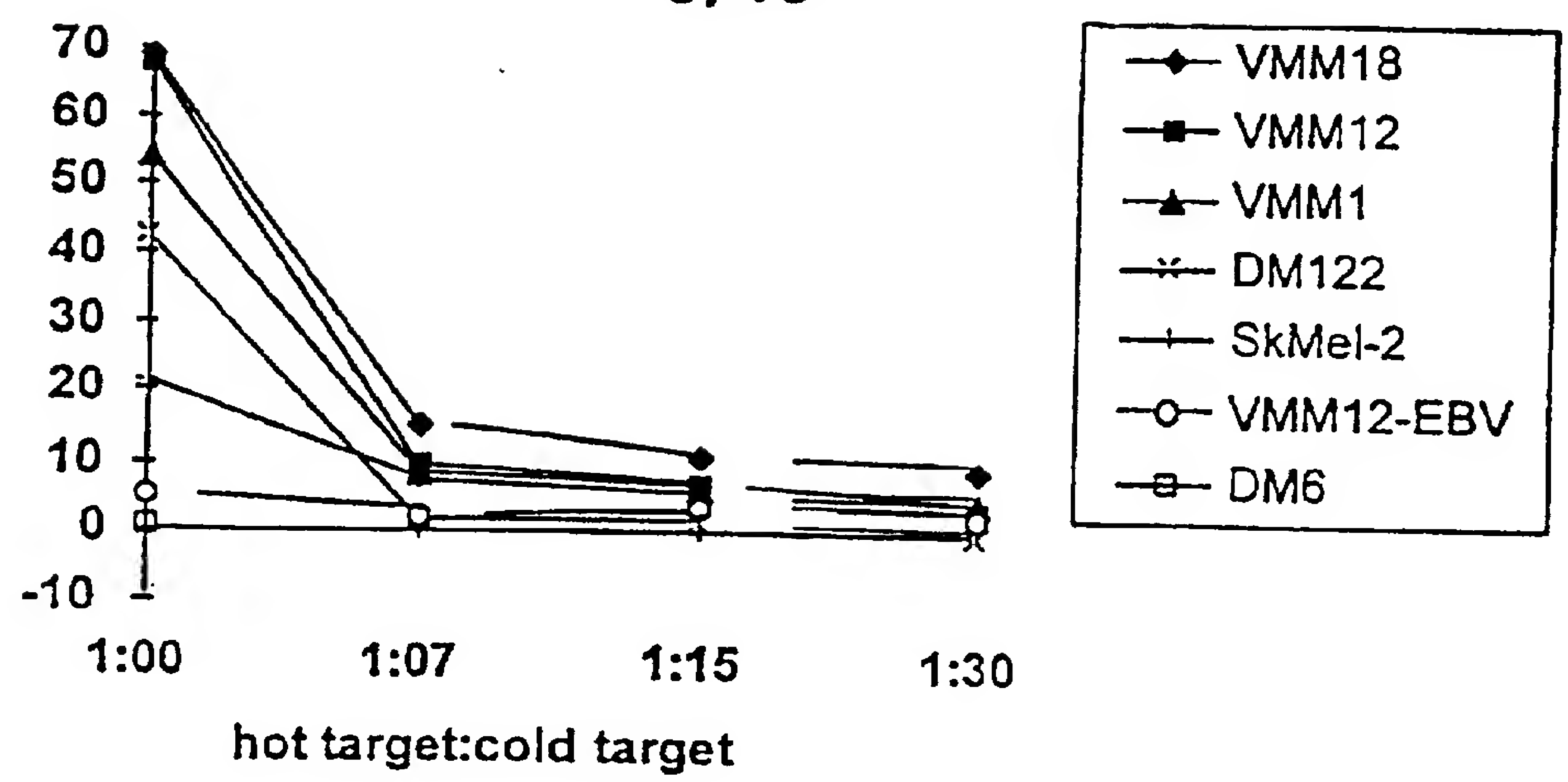


Figure 1B Recognition of VMM18 melanoma by VMM18 CTL was restricted by the class I MHC molecule HLA-A3. Lysis of autologous melanoma was inhibited after incubation of target cells with W6/32 (solid diamonds) and GAP-A3 (solid squares) MAbs, specific for class I MHC and HLA-A3 respectively. Incubation with L243 (open circles) had little effect on recognition of autologous melanoma. Specific lysis of autologous melanoma was 65% (dotted line), while lysis of VMM12-EBV was 1.5% (solid line). The effector:target ratio used was 10:1.



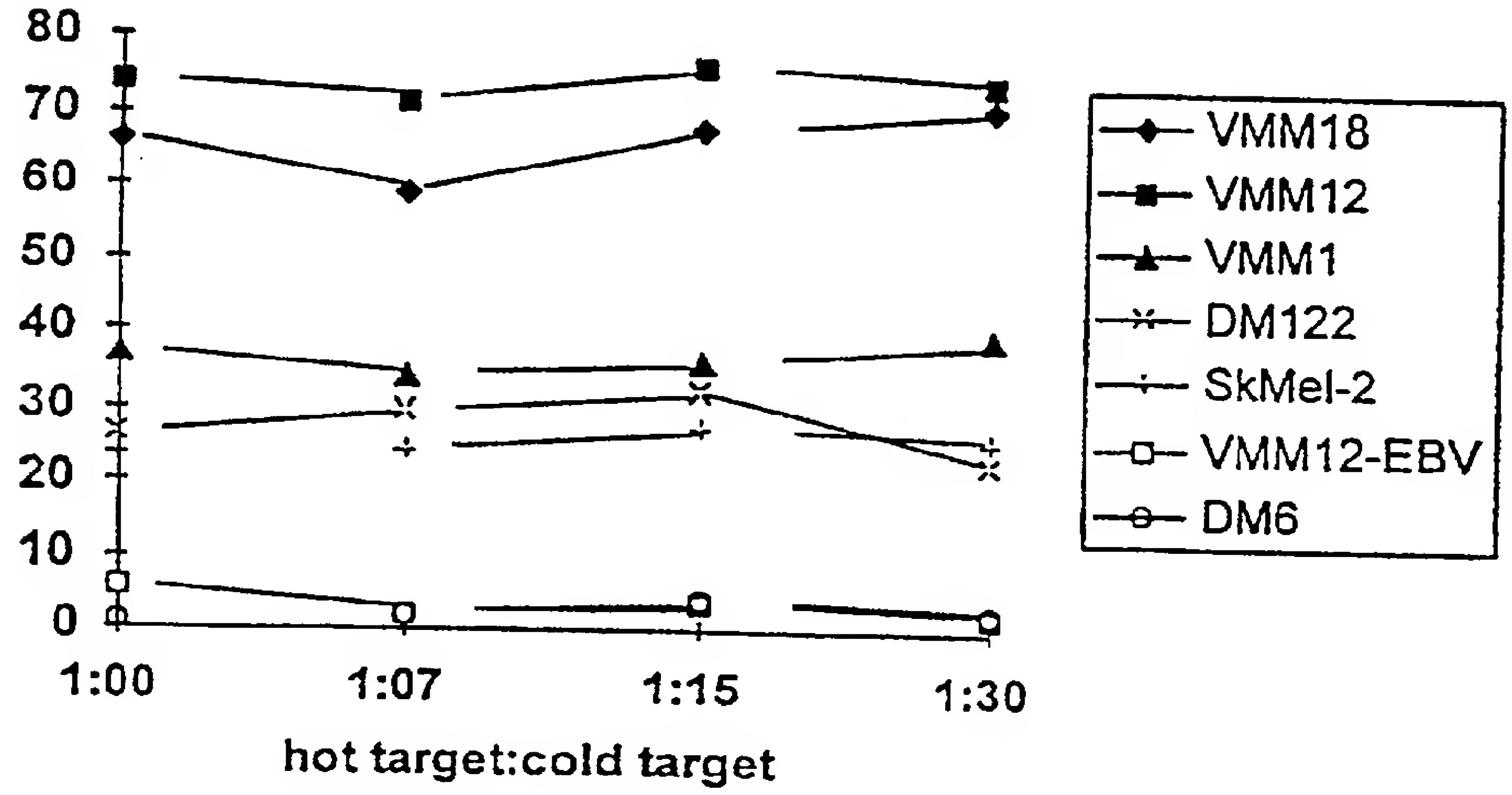


Figure 2 VMM18 CTL recognize a shared antigen expressed by HLA-A3$^{\pm}$ melanomas. Lysis of hot (^{51}chromium labeled) autologous and HLA-A3^{+} allogeneic melanoma cells (see legend) was inhibited by cold (unlabelled) VMM18 melanoma cells (top fig.), but not by cold (unlabelled) HLA-A3^{-} DM6 melanoma cells (bottom fig.). 2×10^4 VMM18 CTL were incubated with 1.4×10^4 unlabelled (cold) VMM18 or DM6 melanoma cells for 1 h at 37^{o}C, prior to the addition of 2×10^3 ^{51}Cr-labelled targets as indicated, giving a final E:T ratio of 10:1.

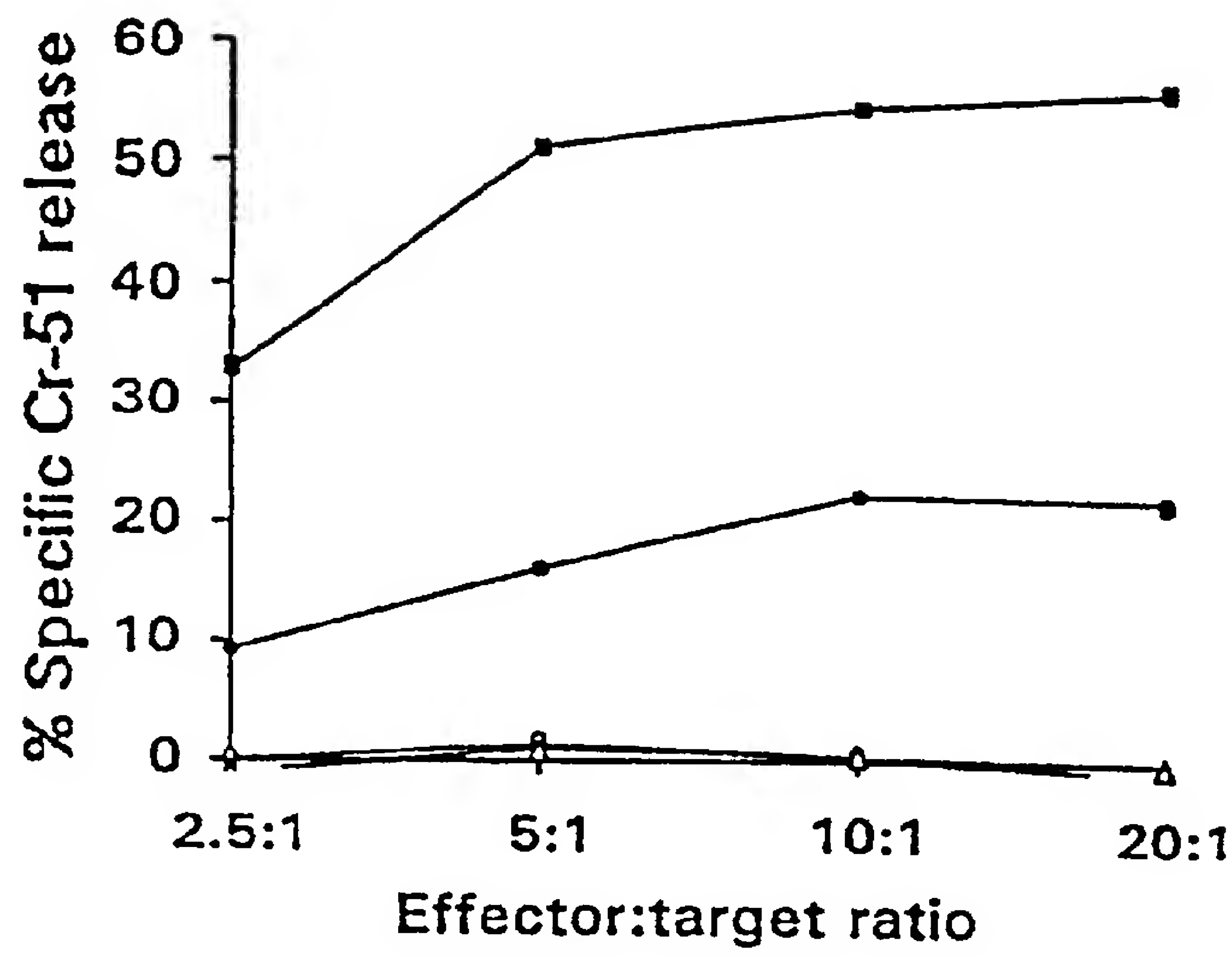


Figure 3 Expression of Pmel-17 reconstitutes recognition of non-melanoma HLA-A3$^{\pm}$ target cells by VMM18 CTL. VMM18 CTL lysed ^{51}Cr-labeled autologous melanoma cells VMM18 (solid squares) as well as a non-melanoma HLA-A3^{+} cell line VMM12-EBV infected with recombinant vaccinia virus expressing Pmel-17 (vac-Pmel-17, closed circles). Minimal lysis of uninfected VMM12-EBV cells (open circles), or cells infected with control recombinant vaccinia virus expressing influenza nucleoprotein (vac-NP, open triangles), was observed.

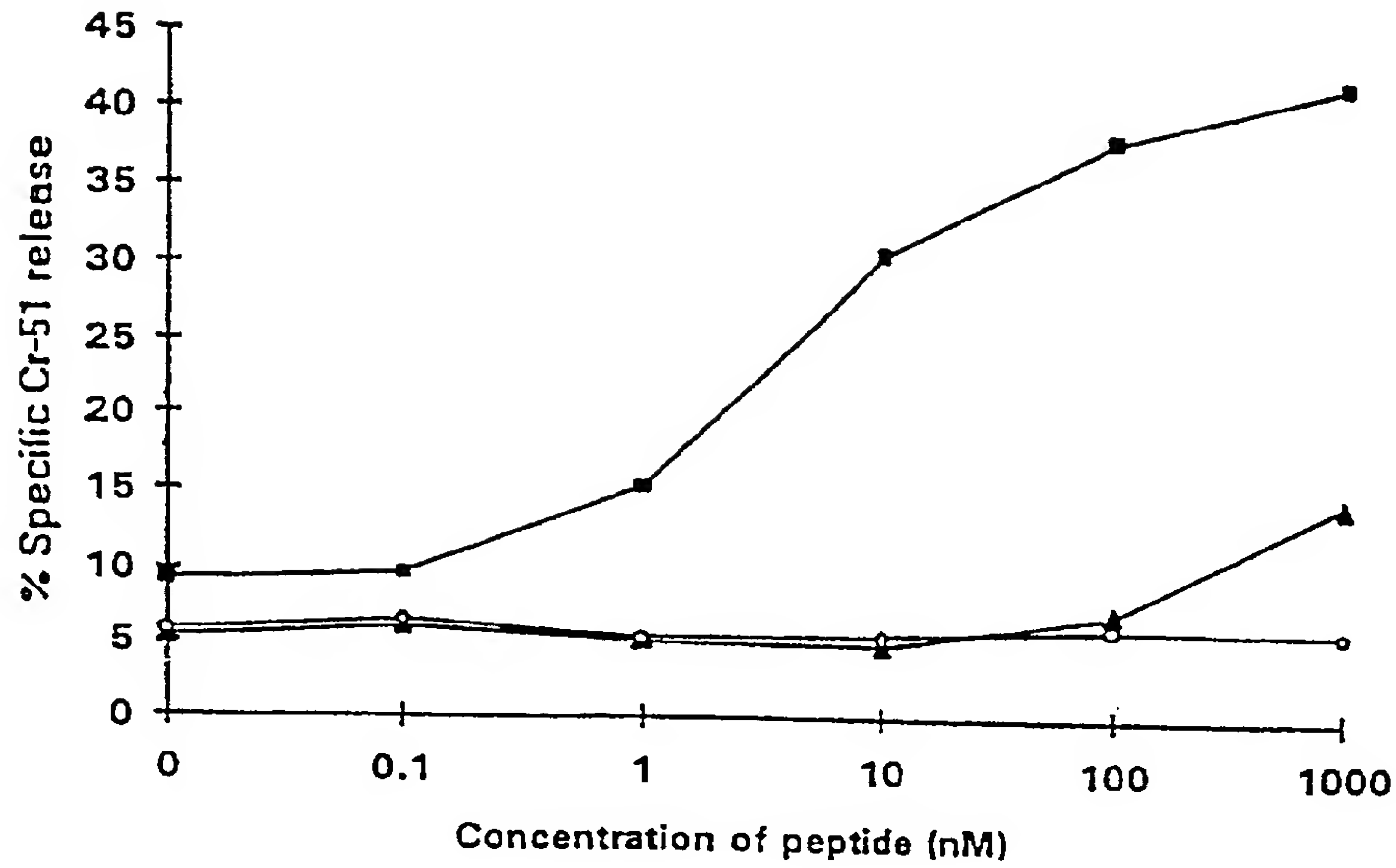


Figure 4 Relative ability of Pmel-17 peptides to sensitize non-melanoma target cells for recognition by VMM18 CTL. ^{51}Cr-labelled T2-A3 cells were incubated with Pmel-17 peptides ALLAVGATK (solid squares) and LLAVGATK (solid triangles) and the control HLA-A3 binding peptide QVPLRPMTYK, from the HIV Nef protein (open circles).

Figure 5A

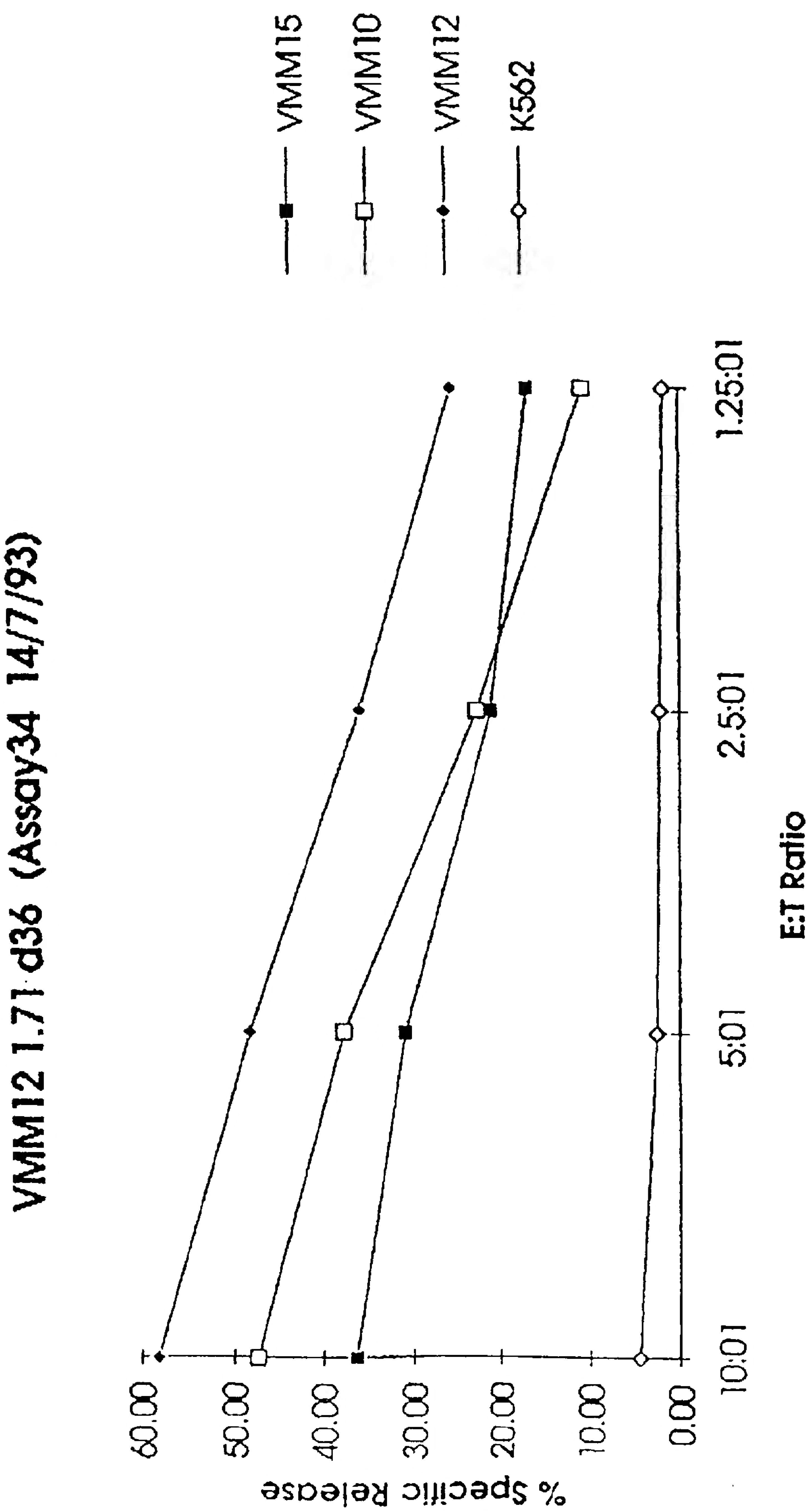

VMM12 1.71 d36 (Assay34 14/7/93)
VMM15
VMM10
VMM12
K562
% Specific Release
60.00
50.00
40.00
30.00
20.00
10.00
0.00
10:01
5:01
2.5:01
1.25:01
E:T Ratio

Figure 5B

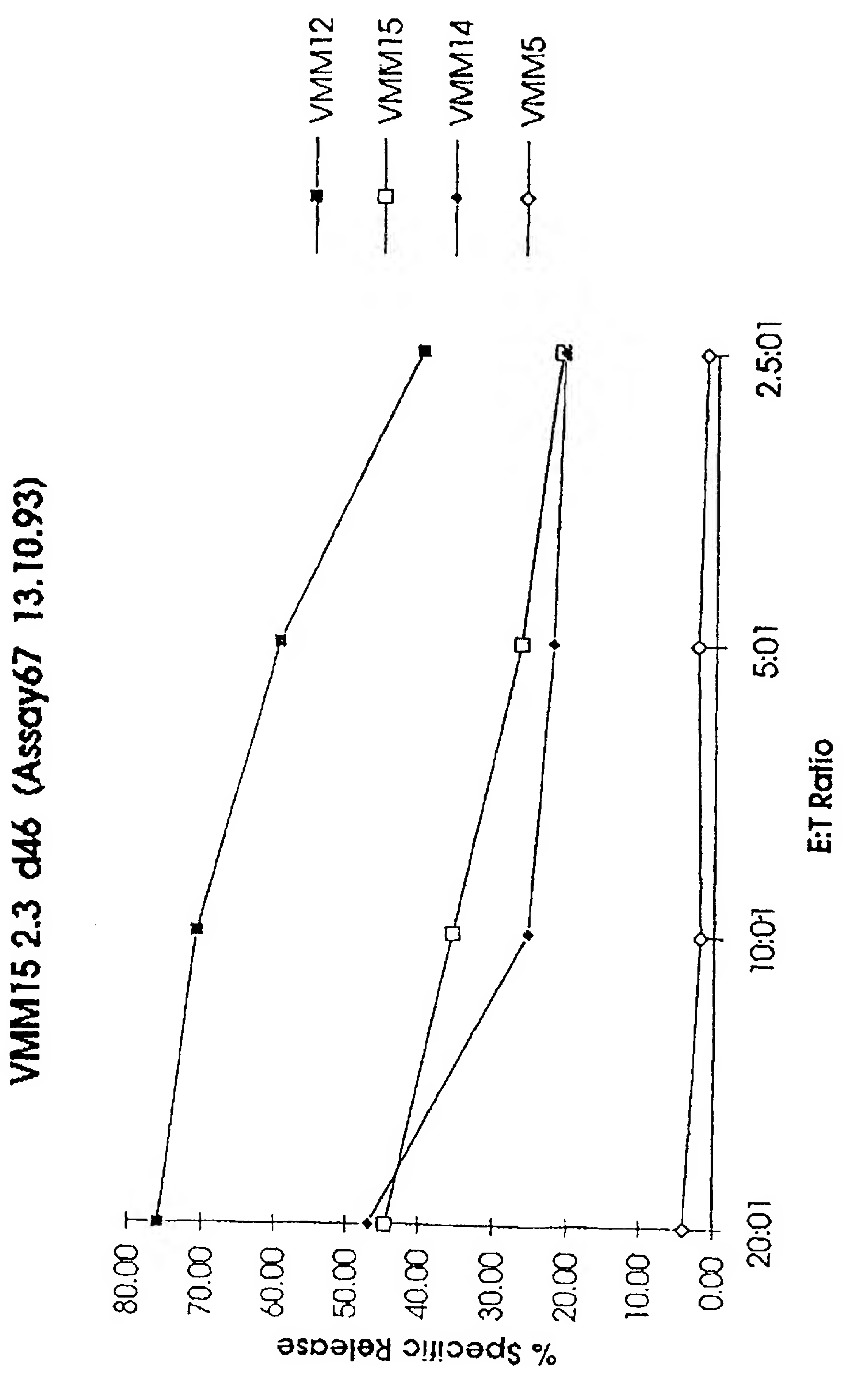

VMM15 2.3 d46 (Assay67 13.10.93)
VMM12
VMM15
VMM14
VMM5
% Specific Release
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
20:01
10:01
5:01
2.5:01
E:T Ratio

Figure 6A

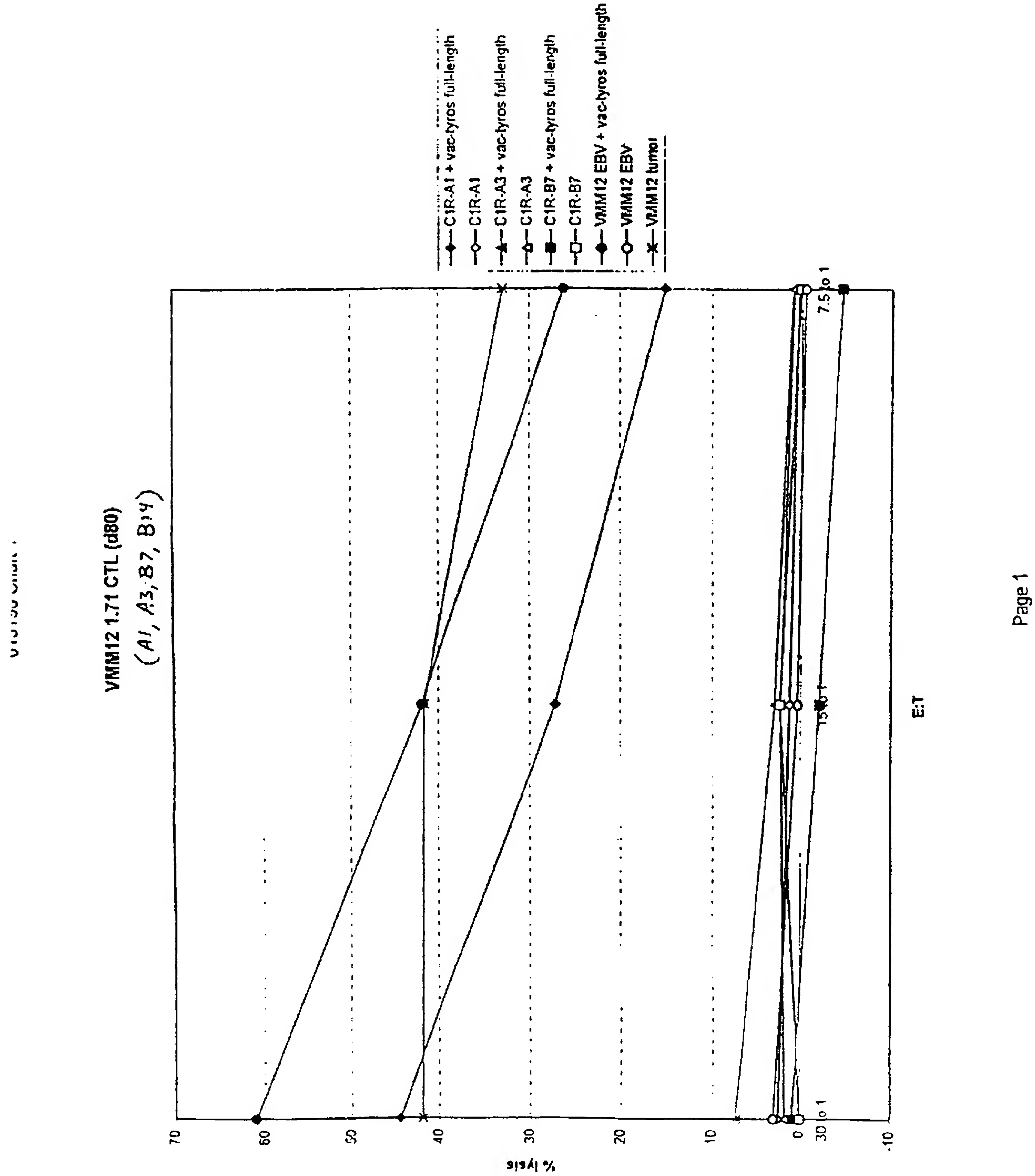

VMM12 1.71 CTL (d80)
(A1, A3, B7, B14)
% lysis
E:T
30 to 1
15 to 1
7.5 to 1
C1R-A1 + vac-tyros full-length
C1R-A1
C1R-A3 + vac-tyros full-length
C1R-A3
C1R-B7 + vac-tyros full-length
C1R-B7
VMM12 EBV + vac-tyros full-length
VMM12 EBV
VMM12 tumor
Page 1

Figure 6B

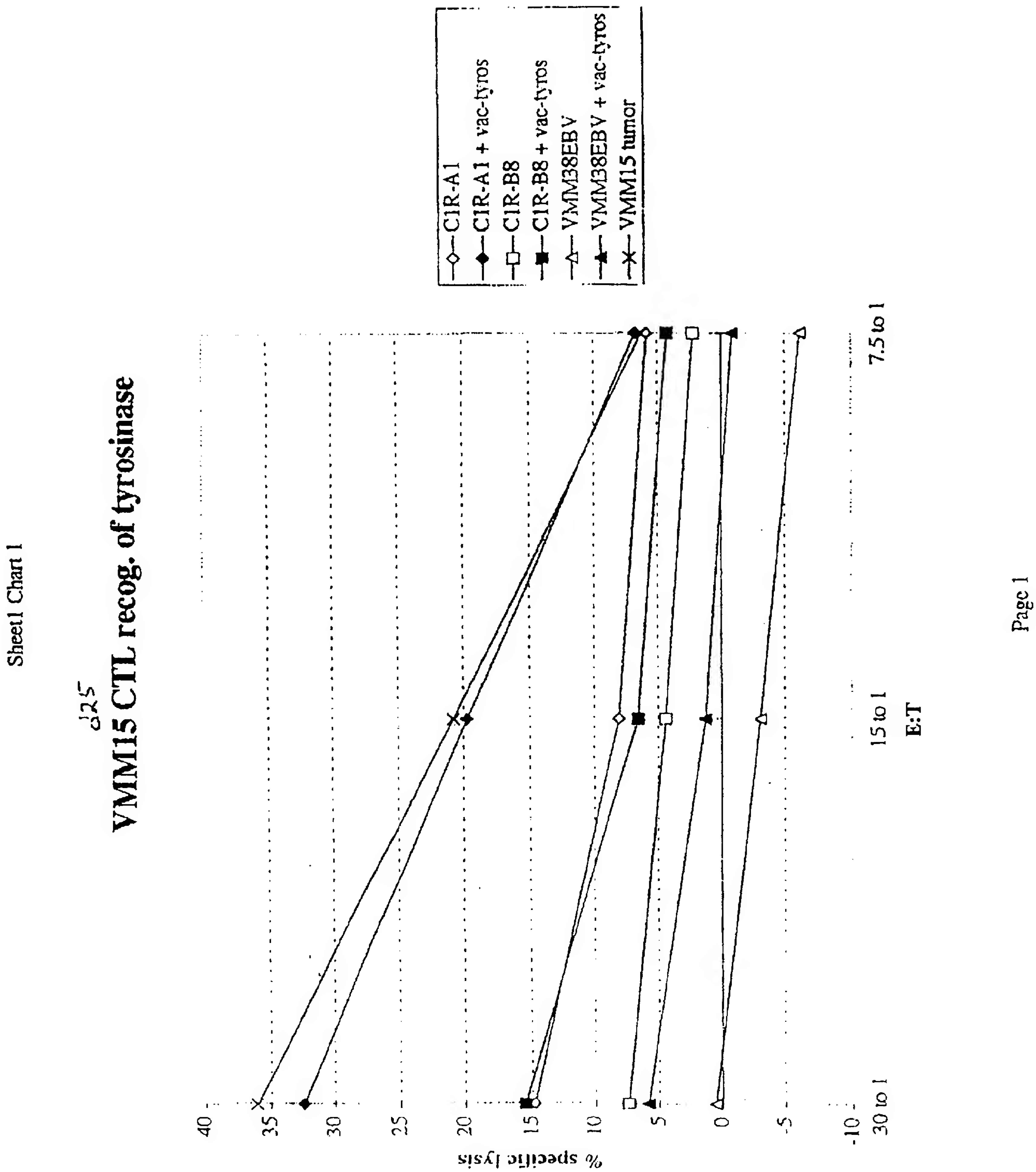

Sheet1 Chart 1
VMM15 CTL recog. of tyrosinase
C1R-A1
C1R-A1 + vac-tyros
C1R-B8
C1R-B8 + vac-tyros
VMM38EBV
VMM38EBV + vac-tyros
VMM15 tumor
% specific lysis
40
35
30
25
20
15
10
5
0
-5
-10
30 to 1
15 to 1
7.5 to 1
E:T
Page 1

Figure 7A

	2/15, 2/22 and 2/29		
1st synthesis	those tested	#?	those in 3Ti
AKHTISSDY	AKHTISSDY	20	AKHTISSDY [illegible]
APEKDKFFAY	APEKDKFFAY	19	
	APVVTHTY	35	
DLFVWIHYY	DLFVWIHYY	22	DLFVWIHYY (diff seq
DLFVWMHIY	DLFVWMHIY	23	DLFVWMHIY (diff sec
DRESWPSVFY	DRESWPSVFY	8	
DSDPDSFQDY	DSDPDSFQDY	3	
DSFQDYIKSY	DSFQDYIKSY	4	
DYVIPIGTY	DYVIPIGTY	21	DYVIPIGTY [illegible]
EFCLSLTQY	EFCLSLTQY	26	EFCLSLTQY 27
EKEDYHSLY	EKEDYHSLY	16	
FISSKDLGY	FISSKDLGY	17	
FQDYIKSY	FQDYIKSY	33	
GDENFTIPY	GDENFTIPY	15	GDENFTIPY [illegible]
ISSKDLGYDY	ISSKDLGYDY	2	
IVCSRLEEY	IVCSRLEEY	25	IVCSRLEEY [illegible]
IYDLFVWMHY	IYDLFVWMHY	5	IYDLFVWMHY [illegible]
KCDICTDEY	KCDICTDEY	11	KCDICTDEY [illegible]
KDLGYDYSY	KDLGYDYSY	29	
KEDYHSLY	KEDYHSLY	34	
PEKDKFFAY	PEKDKFFAY	19	
PIGHNRESY	PIGHNRESY	28	
PLLMEKEDY	PLLMEKEDY	32	
PMFNDINIY	PMFNDINIY	10	PMFNDINIY 11
RESWPSVFY	RESWPSVFY	18	
RHRPLQEVY	RHRPLQEVY	27	
SDPDSFQDY	SDPDSFQDY	30	
SFQDYIKSY	SFQDYIKSY	31	
SKDLGYDY	SKDLGYDY	36	
SKDLGYDYSY	SKDLGYDYSY	7	
SMDALLGGY	SMDALLGGY	24	SMDALLGGY (diff sec
SMHNALHIY	SMHNALHIY	12	SMHNALHIY 13
SSKDLGYDY	SSKDLGYDY	14	
SSMHNALHIY	SSMHNALHIY	1	SSMHNALHIY 1
TGDENFTIPY	TGDENFTIPY	6	TGDENFTIPY 7
YMVPFIPLY	YMVPFIPLY	13	

Figure 7B

and synthesis	the te[illegible]ed	those in 3Ti	
ANAPIGHNRESY	ANAPIG[illegible]NRESY		A
APIGHNRESY	APIGHNRESY		E
DAEKCDICTDEY	DAEKCDICTDEY	DAEKCDICTDEY	П
DLFVWMHYY	DLFVWMHYY	DLFVWMHYY	I\
DPDSFQDYIKSY	DPDSFQDYIKSY		R
DVEFCLSLTQY	DVEFCLSLTQY	DVEFCLSLTQY	T
EKCDICTDEY	EKCDICTDEY	EKCDICTDEY	V
ESYMVPFIPLY	ESYMVPFIPLY		W
FFISSKDLGY	FFISSKDLGY		Y
FISSKDLGYDY	FISSKDLGYDY		Y
GDEDFTIPY	GDEDFTIPY	GDEDFTIPY (n to d)	Y
GSTPMFNDINIY	GSTPMFNDINIY	GSTPMFNDINIY	Y
ISSDYVIPIGTY	ISSDYVIPIGTY	ISSDYVIPIGTY	
ISSKDLGYDYSY	ISSKDLGYDYSY		
IYDLFVWIHY	IYDLFVWIHY	IYDLFVWIHY (seq. diff.)	
IYDLFVWIHYY	IYDLFVWIHYY	IYDLFVWIHYY (seq. diff.)	
IYDLFVWMHYY	IYDLFVWMHYY	IYDLFVWMHYY	
LAKHTISSDY	LAKHTISSDY	LAKHTISSDY (partial)	
LMEKEDYHSLY	LMEKEDYHSLY		
LQDSDPDSFQDY	LQDSDPDSFQDY		
LSAPEKDKFFAY	LSAPEKDKFFAY		
LTGDEDFTIPY	LTGDEDFTIPY	LTGDEDFTIPY (n to d)	
LTGDENFTIPY	LTGDENFTIPY	LTGDENFTIPY	
LTLAKHTISSDY	LTLAKHTISSDY	LTLAKHTISSDY (partial)	
MEKEDYHSLY	MEKEDYHSLY		
PDSFQDYIKSY	PDSFQDYIKSY		
QDSDPDSFQDY	QDSDPDSFQDY		
QIVCSRLEEY	QIVCSRLEEY	QIVCSRLEEY	
QPLLMEKEDY	QPLLMEKEDY		
QRHRPLQEVY	QRHRPLQEVY		
QSSMHNALHIY	QSSMHNALHIY	QSSMHNALHIY	
RESYMVPFIPLY	RESYMVPFIPLY		
RRHRPLQEVY	RRHRPLQEVY		
SDYVIPIGTY	SDYVIPIGTY	SDYVIPIGTY	
SQSSMHNALHIY	SQSSMHNALHIY	SQSSMHNALHIY	
SSDYVIPIGTY	SSDYVIPIGTY	SSDYVIPIGTY	
SSKDLGYDYSY	SSKDLGYDYSY		
STPMFNDINIY	STPMFNDINIY	STPMFNDINIY	
SYMVPFIPLY	SYMVPFIPLY		
TGDEDFTIPY	TGDEDFTIPY	TGDEDFTIPY (n to d)	
TLAKHTISSDY	TLAKHTISSDY	TLAKHTISSDY (partial)	
TPMFNDINIY	TPMFNDINIY	TPMFNDINIY	
VDDRESWPSVFY	VDDRESWPSVFY		
VEFCLSLTQY	VEFCLSLTQY	VEFCLSLTQY	
VSMDALLGGY	VSMDALLGGY	VSMDALLGGY (seq. diff.)	
YVSMDALLGGY	YVSMDALLGGY	YVSMDALLGGY (seq. diff.)	

Figure 7C

ıeet1

3rd synthesis	those tested	those in 3TI
AMERPRDLY		?
EVSTPQILTY		?
ITTACIRAIY		?
IWAMTIAIY		?
RSTTAISLY		?
TTACIRAIY		?
VSTPQILTY		?
WRSTTAISLY		?
YDLFVWIHY		YDLFVWIHY (seq. diff.)
YDLFVWIHYY		YDLFVWIHYY (seq. diff.)
YDLFVWMHY		YDLFVWMHY
YDLFVWMHYY		YDLFVWMHYY

50 µl

Figure 7D

4th synthesis	#	those tested	those in 3T1
ANDPIFLLH	3	ANDPIFLLH	
ANDPIFLLHH	4	ANDPIFLLHH	
CCPPWSGDR	32	CCPPWSGDR	
CTDEYMGGQ	21	CTDEYMGGQ	CTDEYMGGQ
CTDEYMGGQH	24	CTDEYMGGQH	CTDEYMGGQH
CTERRLLVR	2	CTERRLLVR	
CTERRLLVRR	7	CTERRLLVRR	
CVSSKNLMEK	35	CVSSKNLMEK	
DGTPEGPLRR	25	DGTPEGPLRR	DGTPEGPLRR (n to d)
DIDFAHEAPA	36	DIDFAHEAPA	DIDFAHEAPA
DPDSFQDYIK	5	DPDSFQDYIK	
DSDPDSFQD	10	DSDPDSFQD	
DVEFCLSLTQ	8	DVEFCLSLTQ	DVEFCLSLTQ
ECCPPWSGDR	37	ECCPPWSGDR	
FNDINIYDLF	26	FNDINIYDLF	FNDINIYDLF
FTIPYWDWR	12	FTIPYWDWR	FTIPYWDWR
GSEIWRDIDF	1	GSEIWRDIDF	GSEIWRDIDF
GTPEGPLRR	22	GTPEGPLRR	GTPEGPLRR
GYEIWRDIDF	16	GYEIWRDIDF	GYEIWRDIDF (seq. change)
IFDLSAPEK	33	IFDLSAPEK	
LPEEKQPLLM	30	LPEEKQPLLM	
LSAPEKDKF	18	LSAPEKDKF	
LSAPEKDKFF	20	LSAPEKDKFF	
NGDFFISSK	13	NGDFFISSK	
NGTPEGPLRR	27	NGTPEGPLRR	NGTPEGPLRR
QTSAGHFPR	23	QTSAGHFPR	
QYESGSMDK	6	QYESGSMDK	QYESGSMDK
SADVEFCLSL	14	SADVEFCLSL	SADVEFCLSL
SMDKAADFSF	28	SMDKAADFSF	SMDKAADFSF (n to d)
SMDKAANFSF	29	SMDKAANFSF	SMDKAANFSF
SSDYVIPIG	19	SSDYVIPIG	SSDYVIPIG
SSDYVIPIGT	11	SSDYVIPIGT	SSDYVIPIGT
TLEGFASPLT	17	TLEGFASPLT	TLEGFASPLT
YLEQASRIWS	9	YLEQASRIWS	
YMVPFIPLYR	15	YMVPFIPLYR	
YPEANAPIGH	31	YPEANAPIGH	
YWDWRDAEK	34	YWDWRDAEK	YWDWRDAEK

Figure 8

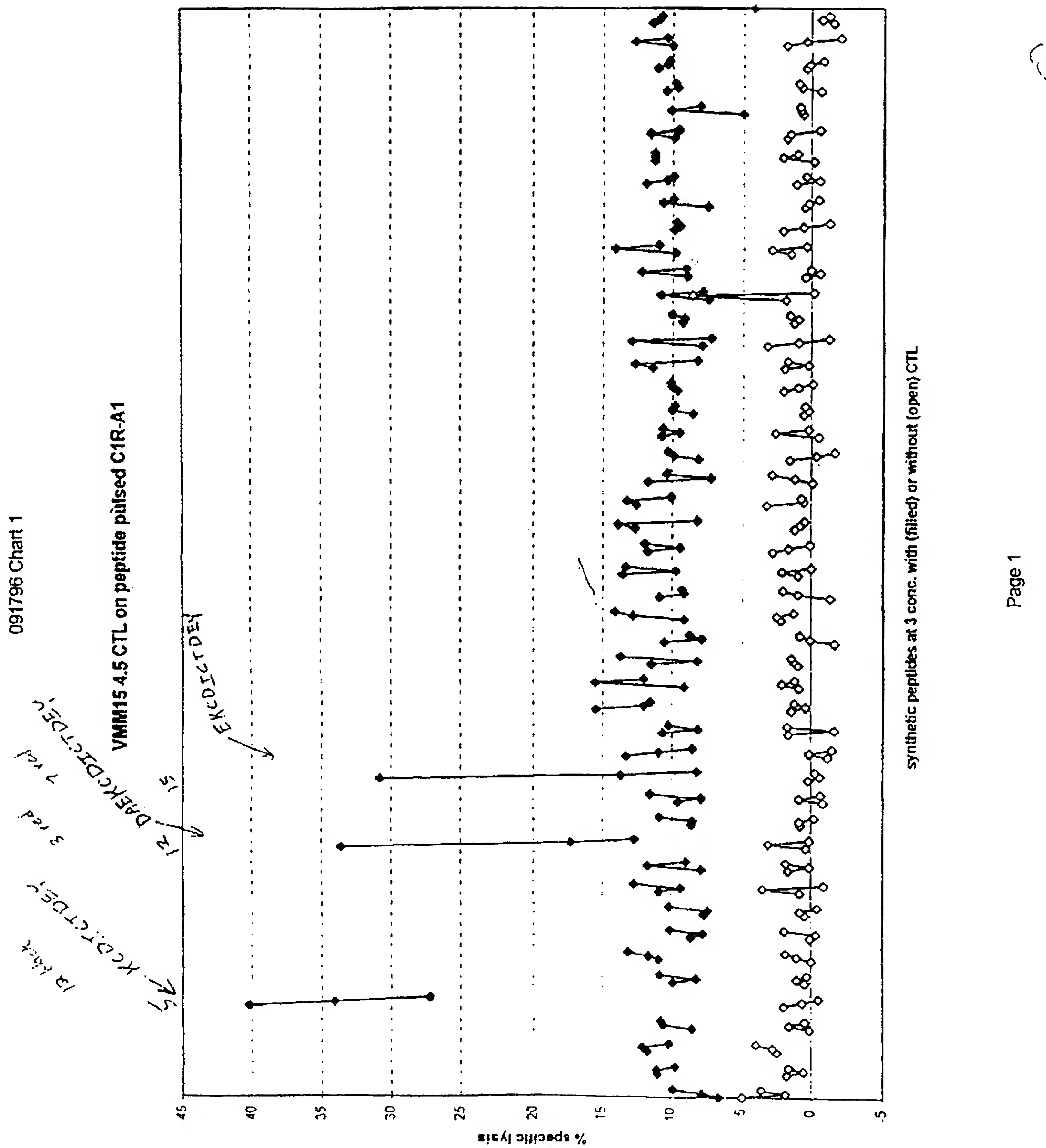

091796 Chart 1
VMM15 4.5 CTL on peptide pulsed C1R-A1
% specific lysis
45
40
35
30
25
20
15
10
5
0
-5
synthetic peptides at 3 conc. with (filled) or without (open) CTL
Page 1

Figure 9

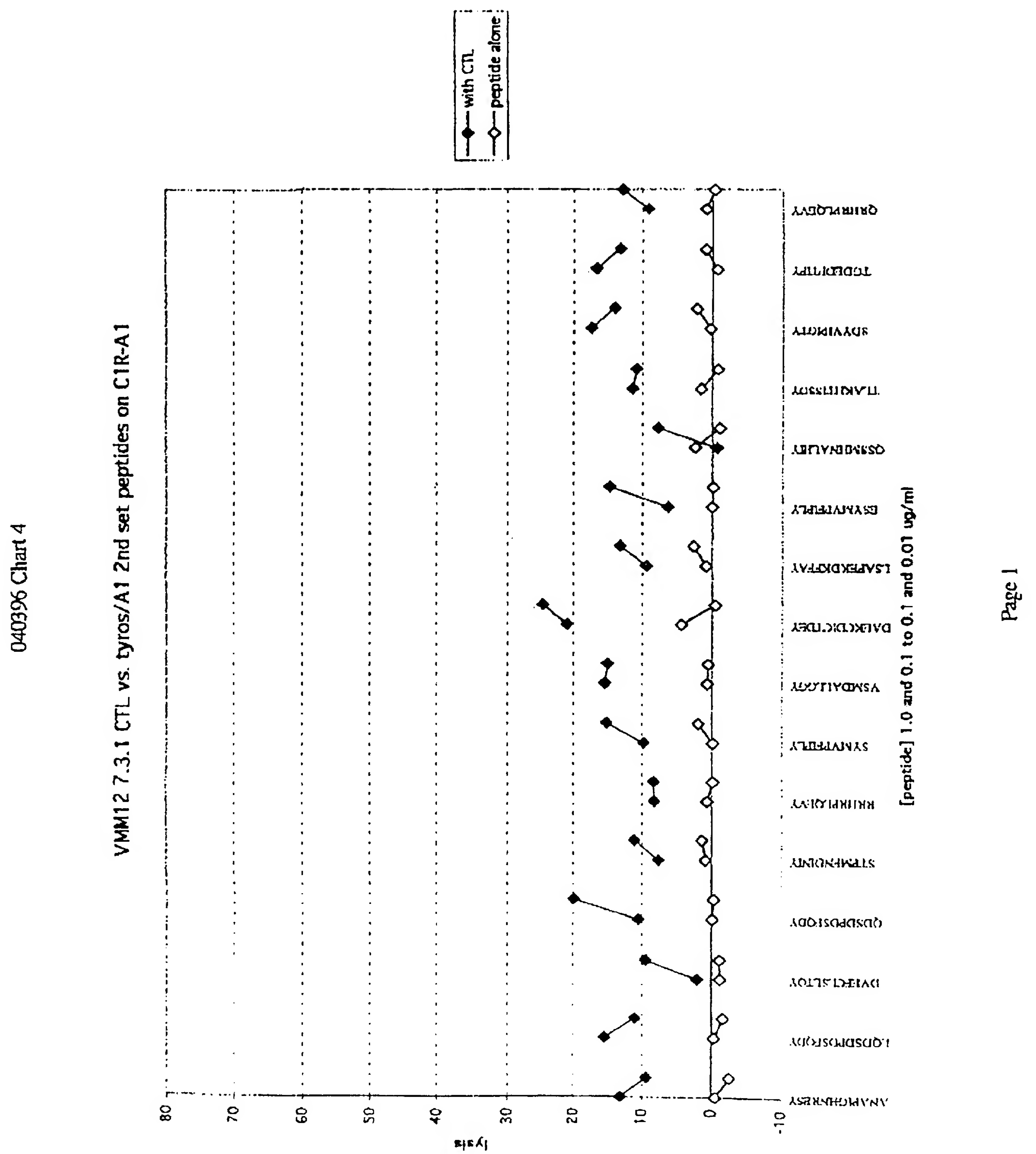

040396 Chart 4
VMM12 7.3.1 CTL vs. tyros/A1 2nd set peptides on C1R-A1
with CTL
peptide alone
lysis
80
70
60
50
40
30
20
10
0
-10
[peptide] 1.0 and 0.1 to 0.1 and 0.01 ug/ml
Page 1

Figure 10

1	MLLAVLYCLL	WSFQTSAGHF	PRACVSSKNL	MEKECCPPWS	GDRSPCGQLS
51	GRGSCQNILL	SNAPLGPQFP	FTGVDDRESW	PSVFYNRTCQ	CSGNFMGFNC
101	GNCKFGFWGP	NCTERRLLVR	RNIFDLSAPE	KDKFFAYLTL	AKHTISSDYV
151	IPIGTYGQMK	NGSTPMFNDI	NIYDLFVWMH	YYVSMDALLG	GSEIWRDIDF
201	AHEAPAFLPW	HRLFLLRWEQ	EIQKLTGDEN	FTIPYWDWRD	AEKCDICTDE
251	YMGGQHPTNP	NLLSPASFFS	SWQIVCSRLE	EYNSHQSLCN	GTPEGPLRRN
301	PGNHDKSRTP	RLPSSADVEF	CLSLTQYESG	SMDKAANFSF	RNTLEGFASP
351	LTGIADASQS	SMHNALHIYM	NGTMSQVQGS	ANDPIFLLHH	AFVDSIFEQW
401	LQRHRPLQEV	YPEANAPIGH	NRESYMVPFI	PLYRNGDFFI	SSKDLGYDYS
451	YLQDSDPDSF	QDYIKSYLEQ	ASRIWSWLLG	AAMVGAVLTA	LLAGLVSLLC
501	RHKRKQLPEE	KQPLLMEKED	YHSLYQSHL		

KCDICTDEY represents residues 243 - 251 of the tyrosinase sequence

17/529 Residues are Cysteine	= 3.2 %	2/9 Residues of KCDICTDEY are Cysteine =	22 %
30/529 Residues are Aspartic acid	= 5.7 %	2/9 Residues of KCDICTDEY are Asp Acid =	22 %
27/529 Residues are Glutamic acid	= 5.1 %	3/9 Residues of KCDICTDEY are Glutamic acid =	11 %
17/529 Residues are Lysine	= 3.2 %	1/9 Residues of KCDICTDEY are Cysteine =	11 %
Total of C-D-E-K	= 17.2 %	=	67%

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/04958

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 31/ 70, 39/00, 39/12, 39/385
US CL : 424/186.1, 193.1, 195.11, 196.11, 198.1, 199.1; 514/25
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/186.1, 193.1, 195.11, 196.11, 198.1, 199.1; 514/25

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

REGISTRY, CA, CAPLUS, BIOSIS, MEDILINE, EMBASE, WPIDS, USPATFULL
search terms: hla-a1, hla-a2, hla-a3, melanoma, pmel-17, gp-100, tyrosinase

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ADEMA et al. Melanocyte Lineage-Specific Antigens Recognized by Monoclonal Antibodies NKI-beteb, HMB-50, and HMB-45 are Encoded by a Single cDNA. Am. J. Path. 6 December, 1993, Vol 143, No. 6, pages 1579-1585, see especially Abstract and pages 1580 and 1583-1584	1-3
X	WO 94/14459 A1 (BOON-FALLEUR ET AL) 07 July 1994, see entire document	1-3
A	JAEGER ET AL. Generation of Cytotoxic T-Cell Responses with Synthetic Melanoma-Associated Peptides In VIVO: Implications for Tumor Vaccines with Melanoma-Associated Antigens. Int. J. Cancer. 1996, Vol 66, pages 162-169, see entire document	1-3

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 JUNE 1997	18 AUG 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer SUSAN UNGAR Telephone No. (703) 305-0196

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SLINGHOFF et al. Recognition of Human Melanoma Cells by HLA-A2.1-Restricted Cytotoxic T Lymphocytes is Mediated by at Least Six Shared Peptide Epitopes. J. Immunol. 1993, Vol. 150, No. 7, pages 2955-2963, see entire document.	2, 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/04958

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 8-22
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
1-22 (Species A-C)

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.